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Carcinogenic Action of Two Azo Compounds in Mice

By HOWARD B. ANDERVONT, *principal biologist*, and JESSE E. EDWARDS, *research fellow*, *National Cancer Institute, National Institute of Health, United States Public Health Service*

Previous experiments (*1*, *2*) showed that the subcutaneous administration of crystalline *o*-aminoazotoluene moistened with glycerol to mice of various inbred strains and their hybrids induced hepatic lesions and hepatomas, and that females were more susceptible than males. This compound also induced pulmonary tumors in certain strains and hemangio-endotheliomas in all strains. In view of these findings, it was considered of interest to ascertain (1) whether similar results attend subcutaneous administration of *o*-aminoazotoluene dissolved in olive oil and (2) whether mice exhibit a sex difference in susceptibility to hepatic changes induced by *p*-dimethylaminoazobenzene which is known (*3*) to produce hepatic lesions in rats.

MATERIALS AND METHODS

Mice of strains C3H, C, A, and C57 Black were used. Those of strain A were procured from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine. The others were obtained from stocks maintained at the National Cancer Institute. The animals were approximately 3 months of age when given the first injection and were maintained under similar environmental conditions, with an unlimited supply of drinking water and Purina dog chow pellets. All were kept as virgins.

Necropsies were performed on all mice. The macroscopic appearance of the liver was noted and a portion of each prepared for histologic examination. The lungs were examined for the presence and number of pulmonary tumors. The occurrence of hepatic lesions, hepatomas, hemangio-endotheliomas, and fibrosarcomas was confirmed microscopically.

EXPERIMENTAL PROCEDURE AND RESULTS

All experiments were begun during June 1941. In the first experiment, olive-oil solutions of *o*-aminoazotoluene or *p*-dimethylaminoazobenzene were administered. Two lots of *o*-aminoazotoluene were used: One was obtained from the Eastman Kodak Co. and was used without further purification; the other was purified (m. p. 102.5°–103° C. corrected) by Dr. J. L. Hartwell, of the National Cancer Institute. The lots gave similar results, and the findings are combined. The *p*-dimethylaminoazobenzene (m. p. 117°–118° corrected) was also supplied by Dr.

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Hartwell. The olive oil was of U. S. P. quality.

Solution of 20 mg. of either compound in 1 cc. of olive oil was accomplished by heating gently so that the flask was not too warm to hold in the palm of the hand. Fresh solutions were prepared for each monthly injection.

All injections were made subcutaneously into the right axillary region with a 1-cc. syringe, and each consisted of 0.25 cc. of olive oil containing 5 mg. of compound. Each animal received nine injections at monthly intervals, or a total of 45 mg. of compound. All surviving mice were killed 1 year after receiving the initial injection.

The *o*-aminoazotoluene was administered to mice of strains C, C57 Black, C3H, and A. The induction of hepatic lesions is summarized in table 1. The gross and microscopic appearance of the hepatic changes were described in a previous report (*2*). Seventy-five percent of strain C females and seventy-six percent of strain C57 Black females showed gross hepatic lesions, whereas none of the males showed any reaction. All the strain C3H females developed spontaneous mammary tumors before the end of the experiment; but of two which survived for 51 weeks after the first injection, one exhibited a reaction. None of the C3H males developed a liver lesion. Seven strain A females killed at the termination of the experiment showed definite hepatic reactions. The male strain A animals died of an intercurrent infection shortly after the beginning of the investigation. None of the other mice died before the twenty-eighth week.

Hepatomas were not observed in any animal coming to necropsy before the fifty-second week; but of the mice killed when the experiment was ended, 10 strain C females, 5 strain C57 Black females, 4 strain C3H males, and 2 strain A females had developed hepatomas. The occurrence of this neoplasm in 44 percent of 15 month-old strain C3H males is in accordance with the observed incidence (*1*) in normal mice of this strain.

Pulmonary tumors occurred in mice of strains C and A only. Of 33 strain C mice killed at the end of the experiment, 14, or 42 percent, had pulmonary tumors, and of the 7 strain A mice 6, or 85 percent, developed this neoplasm.

Ten mice developed hemangio-endotheliomas. The occurrence of these tumors is summarized in table 2. The gross and microscopic appearance of this type of tumor was also described in the earlier paper (*2*). One tumor arose in the right axillary region (injection site) of a strain C male.

Fibroscarcomas occurred at the site of

TABLE 1.—*The induction of macroscopic hepatic changes in mice of strains C, C57 Black, C3H, and A following subcutaneous administration of* o-*aminoazotoluene dissolved in olive oil*

[Numerator = mice killed
Denominator = mice with hepatic changes]

Strain	Sex	Number of mice	Time in weeks										Total	Incidence of hepatic changes
			28	33	37	41	42	46	49	50	51	52		
														Percent
C	F	20			4/0							16/15	20/15	75
C	M	20	1/0	1/0			1/0					17/0	20/0	0
C57 Black	F	21				2/0			1/1	1/1	1/1	16/13	21/16	76
C57 Black	M	20			1/0		2/0		1/0		1/0	15/0	20/0	0
C3H	F	8		2/0	1/0		2/0	1/0			2/1		8/1	12
C3H	M	9										9/0	9/0	0
A	F	8	1/0									7/7	8/7	87

TABLE 2.—*Occurrence of hemangio-endotheliomas in mice of strains C and C57 Black following subcutaneous administration of o-aminoazotoluene dissolved in olive oil*

Strain	Sex	Number of mice	Time after first injection	Site of origin
			Weeks	
C	F	3	52	Interscapular fat.
C	F	1	52	Ovary.
C	M	1	42	Right axilla.
C	M	1	52	Interscapular fat.
C57 Black	F	1	51	Liver.
Do	F	1	52	Interscapular fat.
Do	M	1	51	Do.
Do	M	1	52	Do.

subcutaneous injection (right axilla) in five mice. Two were found in strain C57 Black females 41 weeks after the first injection; one occurred in a strain C3H female during the forty-second week; one occurred in a strain C57 Black female during the fiftieth week; and one was found in a strain C female killed at the conclusion of the experiment.

The foregoing results show that the subcutaneous injection of olive-oil solutions of *o*-aminoazotoluene into mice was followed by hepatic lesions, hepatomas, pulmonary tumors, hemangio-endotheliomas, and fibrosarcomas.

The *p*-dimethylaminoazobenzene was administered to mice of strain C (17 females and 17 males), strain C57 Black (18 females and 14 males), and strain A (10 females and 9 males). Of these mice, 16 strain C females, 16 strain C males, 10 strain C57 Black females, 8 strain C57 Black males, 2 strain A females, and 4 strain A males were alive 1 year after the first injection. With the exception of 8 strain C females and 8 strain C males, all were killed and necropsied. None of the animals dying or killed during the course of the experiment had gross or microscopic hepatic changes. The strain C mice were kept to ascertain whether the compound induced hepatic reactions or tumors if the animals were permitted to live their normal life span.

None of the mice developed pulmonary tumors or hemangio-endotheliomas. Two strain C57 Black females coming to necropsy during the forty-first week of the experiment had fibrosarcomas in the right axilla.

As controls five strain C females, four strain C males, four strain C57 Black females, and four strain C57 Black males received subcutaneous injections of 0.25 cc. of olive oil each time injections were given to the experimental animals. The olive oil was heated in exactly the same manner as that used as solvent. All the mice lived to the conclusion of the experiment, and none developed a lesion of any kind.

In the second experiment mice of strain A (10 males and 10 females) and strain C (16 males and 14 females) were given subcutaneous injections of crystalline *p*-dimethylaminoazobenzene moistened with glycerol into the right axilla. The substance was administered according to the technique described by Shear (*5*). Six drops of glycerol was added to each 200 mg. of the compound. Each mouse received 10 injections of approximately 10 mg. at monthly intervals, a total of 100 mg.

Ten months after the first injection five strain A females, four strain A males, four strain C females, and four strain C males were killed and necropsied. The organs of all mice were normal. Two months later all surviving mice, with the exception of five strain C females, were killed. None had gross or microscopic hepatic lesions. The five strain C females were kept to find out whether hepatic changes would occur at a later date.

Pulmonary tumors were found in the lungs of 2 of 9 strain A mice killed 10 months after the first injection and in 4 of

11 killed 2 months later. Thus, of 20 strain A mice, 6, or 30 percent, developed pulmonary tumors. This incidence is considered normal in 12-to-15-month-old mice of this strain (*6*). Pulmonary tumors were the only neoplasm noted in any mouse of the experiment.

The subcutaneous administration of 100 mg. of *p*-dimethylaminoazobenzene moistened with glycerol failed to evoke hepatic changes or tumors in mice of strains A and C.

DISCUSSION

The results of other experiments (*2*) and those presented here show that in mice the subcutaneous administration of *o*-aminoazotoluene moistened with glycerol or dissolved in olive oil induces hepatic reactions, hepatomas, pulmonary tumors, and hemangio-endotheliomas. The compound evokes similar lesions and tumors when injected in solution or as crystals.

The production of hepatic lesions and hepatomas by subcutaneous injections of *o*-aminoazotoluene in solution is in accord with the findings of Yoshida (*7*), Morosenskaya (*8*), Law (*9*), and Turner and Mulliken (*10*). Yoshida and Law used olive oil as the solvent, Morosenskaya used sunflower-seed oil, while Turner and Mulliken employed corn oil. The data are insufficient to determine the influence of the various solvents upon the activity of the compound or to compare its relative carcinogenic action in the solid state or in solution.

Morosenskaya and Law, as well as Turner and Mulliken, used male and female mice but did not record a sex difference in susceptibility to hepatic lesions. In the experiment reported here, females of strains C and C57 Black were more susceptible than males. This result is similar to that of previous experiments (*2*) in which mice of strains A, C, and C57 Black received subcutaneous injections of glycerol-moistened *o*-aminoazotoluene. Experiments are in progress to ascertain whether mice exhibit a sex difference in susceptibility to the compound when it is incorporated into the diet.

The occurrence of subcutaneous tumors at the site of administration of a solution of *o*-aminoazotoluene confirms the work of Law, and of Turner and Mulliken. Law's results may be compared with the present findings since C57 Black mice were used in both studies. In the experiment recorded here, a total of 45 mg. was injected into each of 41 animals, and 3, or 7 percent, developed fibrosarcomas at an average age of 44 weeks, whereas in Law's experiment a total of 10 mg. administered to each of 30 mice produced similar neoplasms in 13, or 43 percent, at an average age of 37 weeks. The higher incidence in Law's animals may have been attributable to a variety of factors such as dosage, technique of administration, or substrains of mice. Turner and Mulliken induced 8 sarcomas in 10 strain C mice which had received 2.5 mg. of the compound each week for more than 1 year.

The production of tumors at the site of administration of solutions of *o*-aminoazotoluene is of special interest, for up to the present time tumors have not appeared at the site of injection of the crystalline compound. This indicates that *o*-aminoazotoluene is more carcinogenic for subcutaneous tissues when in solution and also shows that the carcinogenic activity is not limited to tissues distant from the site of administration. The compound is now known to induce hepatomas, pulmonary tumors, and subcutaneous fibrosarcomas as well as hemangio-endotheliomas in the ovary, lung, liver, adrenal cortex, and in fat depots throughout the body of the

mouse. Morosenskaya also reports the production of mammary tumors and lymphosarcomas of the thymus in mice.

Under the conditions of the present experiments, *p*-dimethylaminoazobenzene was far less carcinogenic for the mouse than was *o*-aminoazotoluene. The former was used to ascertain whether mice exhibit a sex difference in susceptibility to its carcinogenic activity, but 45 mg. dissolved in olive oil or 100 mg. moistened with glycerol did not induce hepatic changes or tumors remote from the injection site. This is in striking contrast with the response of mice of the same strains to similar amounts of *o*-aminoazotoluene as recorded here and elsewhere (*2*). In Law's experiments *p*-dimethylaminoazobenzene was less active in producing hepatic lesions than was *o*-aminoazotoluene. Of 30 strain dba mice receiving *o*-aminoazotoluene, 7 developed hepatic reactions; whereas of the same number receiving *p*-dimethylaminoazobenzene, only 1 had a hepatic lesion. Kinosita (*3*) states: "Butter yellow in olive-oil was injected into mice, no successful result has yet been obtained." Shear (*5*) induced hepatomas in mice of strains L and A by administering 60 mg. of glycerol-moistened *o*-aminoazotoluene, but Shear and Stewart [1] gave 70 mg. of crystalline *p*-dimethylaminoazobenzene to male mice of strains A and dba with negative results although the experiment lasted for 15 months.

However, the subcutaneous injection of 45 mg. of *p*-dimethylaminoazobenzene produced fibrosarcomas at the injection site in 2 strain C57 Black females. Law observed the same type of tumor in 6 of 29 C57 Black mice following subcutaneous administration of 10 mg. of the compound and concluded that in this respect, also, *o*-aminoazotoluene is a more potent carcinogen than is *p*-dimethylaminoazobenzene. His observations, together with those presented here, suggest that in the mouse olive-oil solutions of *p*-dimethylaminoazobenzene may be more carcinogenic for subcutaneous tissues adjacent to the injection site than for tissues at remote sites. The *o*-aminoazotoluene, however, may be more carcinogenic for remote tissues, according to the experiment described herein.

The differences in the susceptibility of mice to the carcinogenic activities of *o*-aminoazotoluene and *p*-dimethylaminoazobenzene, together with the sex factor in susceptibility to hepatic lesions induced by *o*-aminoazotoluene, offer an opportunity for studies of the mechanism of tumor formation by these compounds.

[1] *Shear, M. J., and Stewart, H. L.: Unpublished work from this Institute. See Hartwell* (11).

SUMMARY

Olive-oil solutions of *o*-aminoazotoluene or *p*-dimethylaminoazobenzene were administered subcutaneously to highly inbred strains of mice.

The *o*-aminoazotoluene induced hepatic changes and hepatomas in females of strains C, C57 Black, C3H, and A but did not produce gross or microscopic hepatic reactions in males of strains C, C57 Black, or C3H. Pulmonary tumors were induced in strains A and C. Hemangio-endotheliomas were induced in strains C and C57 Black. Fibrosarcomas were evoked at the site of injection in strains C57 Black, C3H, and C. With the exception of the occurrence of tumors at the site of application, these results were similar to those obtained previously following subcutaneous administration of the crystalline compound moistened with glycerol.

The *p*-dimethylaminoazobenzene did not produce any gross or microscopic

hepatic changes in mice of strains C, C57 Black, or A. The only neoplasms induced were fibrosarcomas at the site of injection in two strain C57 Black females.

Crystalline *p*-dimethylaminoazobenzene moistened with glycerol was administered subcutaneously to mice of strains A and C and did not produce hepatic lesions or neoplasms of any type.

In these experiments *o*-aminoazotoluene was a more potent carcinogen than was *p*-dimethylaminoazobenzene.

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Response of Strain A Female Mice to Small Amounts of *o*-Aminoazotoluene

By Howard B. Andervont, *principal biologist*, and Jesse E. Edwards, *research fellow*, *National Cancer Institute, National Institute of Health, United States Public Health Service*

In earlier experiments (*1*, *2*) inbred strains of mice and their hybrids were given subcutaneous injections of crystalline *o*-aminoazotoluene moistened with glycerol. Each mouse received approximately 10 mg. of the compound monthly for 10 or 11 successive months, or a total of 100 to 110 mg. The experiments were terminated 1 year after the first injection, when all surviving mice were killed. Any animal dying during the course of the experiments was necropsied, the degree of hepatic change noted, and a portion of the liver studied microscopically. It was found that female mice are far more susceptible to hepatic lesions induced by the compound than are males. A small number of females died from 10 to 30 weeks after the first injection, and none showed hepatic changes; but those necropsied after the thirtieth week had developed definite hepatic lesions. The macroscopic and microscopic appearance of these lesions were described in a previous publication (*2*). Grossly, the liver, which was cirrhotic in appearance, was enlarged and often contained hepatomas. The microscopic changes consisted of a variable degree of endothelial proliferation and an increase in reticulum.

The occurrence of hepatic lesions in the females before they had received all the injections indicated that a total of 100 mg. of the compound was not essential for their production. An experiment was therefore made to ascertain whether smaller quantities of *o*-aminoazotoluene induce hepatic reactions and tumors in females of a highly inbred strain of mice. The present paper deals with the results of this experiment.

Female mice of strain A were selected as test animals. From the publication of Cloudman, Bittner, and Little (*3*) and experience in this laboratory, it was known that these mice develop few spontaneous hepatomas but are susceptible to hepatic lesions induced by subcutaneous administration of *o*-aminoazotoluene. Furthermore, earlier work had shown them to be susceptible to the development of pulmonary tumors (*4*) and hemangio-endotheliomas (*2*) following subcutaneous injection of this compound.

The mice were obtained from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine, and were approximately 2½ months old at the beginning of the experiment. They were maintained on a diet of Purina dog chow pellets and were given an unlimited supply of water.

One hundred and forty-four mice were used. Of these, 120 were given *o*-aminoazotoluene, and the remaining 24 were kept as untreated controls. The first treatment was given during March 1941 and consisted of the subcutaneous administration into the right axillary region of 10 mg. of the compound moistened with glycerol (6 drops of glycerol to 200 mg. of *o*-aminoazotoluene). Twenty mice, designated as group 1, did not receive further injections. During April another injection was given to each of the remaining 100 mice, of which 20 were designated as group 2 and set aside without further injection. This procedure occurred at monthly intervals for 6 months and

resulted in 6 groups of animals which had received 10, 20, 30, 40, 50, and 60 mg. of the compound, respectively. Six months after the initial injection, or 1 month after the last injection, 5 mice from each group were killed. Three months later more animals from each group were killed. All surviving mice were killed 1 year after receiving the first injection.

Every mouse killed or dying was necropsied. The appearance of the liver was noted, and sections were prepared for histologic study; the lungs were examined for pulmonary tumors, and other organs were examined for gross changes. All gross changes, as well as the occurrence of hepatomas and hemangio-endotheliomas, were confirmed by histologic examination.

RESULTS

Gross hepatic changes were seen in every mouse, and the degree of reaction appeared to be proportional to the amount of compound administered. The difference in severity of hepatic changes was especially noticeable when mice of different groups were killed during the same day. The livers of those of group 1 (10-mg. dose) killed 6 months after injection were definitely cirrhotic and slightly enlarged, whereas the livers of group 6 animals (60-mg. dose) were markedly enlarged and roughened and contained hepatomas. As the experiment progressed the severity of hepatic lesions increased in all groups. The occurrence of hepatoma is summarized in table 1.

Table 2 gives the incidence of pulmonary tumors and hemangio-endotheliomas. The average number of pulmonary tumors in positive lungs ranged from one in the controls to three in the animals receiving either 50 or 60 mg. of the carcinogen. Hemangio-endotheliomas were found in all groups except group 1 and the control group. Sixteen were found and were distributed as follows: seven in the interscapular fat; six in peritoneal fat deposits; one in the subcutaneous fat; two in the ovary. This induced neoplasm was described in the previous paper (*2*).

DISCUSSION

The experiment confirmed previous findings in that subcutaneous administration of crystalline *o*-aminoazotoluene moistened with glycerol induces hepatic lesions, hepatomas, pulmonary tumors, and hemangio-endotheliomas in susceptible mice. A single injection of 10 mg. of the compound induced hepatic reactions within 27 weeks and hepatomas within 35 weeks. This is of interest in view of the earlier work (*2*) which revealed a striking sex difference in susceptibility to induced

TABLE 1.—*Induction of hepatomas in strain A female mice following subcutaneous administration of 10 to 60 mg. o-aminoazotoluene*

[Numerator = mice killed
Denominator = mice with hepatomas]

Group	Number of mice	Amount administered	Time (in weeks)														Total	Incidence of hepatomas
			27	29	35	37	39	40	41	42	43	44	45	46	48	52		
		Mg.																*Percent*
1	20	10	5/0	....	1/1	....	....	5/2	....	....	....	....	1/0	1/0	....	7/3	20/6	30
2	20	20	5 0	1/0	1/1	....	....	6/2	4/2	1/0	....	....	2/1	....	....	....	20/6	30
3	20	30	5/1	....	....	....	....	5/4	....	5/1	2/1	....	3/1	....	....	....	20/8	40
4	20	40	5/2	....	1/0	....	1/1	5/3	....	....	....	2/1	6/3	....	....	....	20/10	50
5	20	50	5/2	....	1/1	....	2/1	4/3	....	....	....	....	2/1	....	2/1	4/2	20/11	55
6	20	60	5/2	....	....	1/0	....	5/4	....	....	....	1/1	8/6	....	....	....	20/13	65
Control	24	0	....	....	....	....	....	....	....	....	....	....	....	....	....	24/0	24/0	0

TABLE 2.—*Induction of pulmonary tumors and hemangio-endotheliomas in strain A female mice following subcutaneous administration of 10 to 60 mg. o-aminoazotoluene* [1]

[Numerator = mice with pulmonary tumors
Denominator = mice with hemangio-endotheliomas]

Group	Number of mice	Amount administered	Time in weeks													Total	Incidence of pulmonary tumors	Average number of pulmonary tumors in positive lungs	Incidence of hemangio-endotheliomas
			27	29	35	37	39	40	41	42	43	44	45	48	52				
		Mg.															*Percent*		*Percent*
1	20	10	1/0	....	1/0	....	....	3/0	....	....	....	....	1/0	....	6/0	12/0	60	2.0	0
2	20	20	1/0	1/0	1/1	....	....	4/0	1/0	1/0	....	....	2/0	....	....	11/1	55	2.2	5
3	20	30	1/0	....	....	....	....	4/0	....	5/0	2/0	....	3/1	....	....	15/1	75	2.3	5
4	20	40	5/0	....	0/1	....	1/1	5/1	....	....	....	2/0	5/0	....	....	18/3	90	2.1	15
5	20	50	2/0	....	1/1	....	2/2	4/0	....	....	....	....	2/0	2/0	4/2	17/5	85	3.0	25
6	20	60	3/0	....	....	1/1	....	5/2	....	....	....	1/1	7/2	....	....	17/6	85	3.0	30
Control	24	0	....	....	....	....	....	....	....	....	....	....	....	....	7/0	7/0	29	1.0	0

[1] The number of mice killed or dying is given in table 1.

hepatic changes and hepatomas when 100 mg. was administered. All females of strains A, C, and C57 Black developed hepatic reactions, whereas only 45 percent of strain A males, 8 percent of strain C males, and 9 percent of strain C57 Black did so. Since a 10-mg. dose evoked the lesion in all females while 100-mg. doses failed to do so in most males, the sex factor in susceptibility must be pronounced.

Induction of hepatic lesions by a single 10-mg. injection suggests the use of a similar amount to ascertain the relative susceptibilities of different strains of mice. In previous experiments (*1*, *3*) all females of strains A, C, C57 Black, and I developed the lesion following the administration of 100 or 110 mg. The use of 10- or 20-mg. doses may reveal strain differences in susceptibility. These investigations are in progress.

From the indications, the minimum effective dose of the compound in strain A females is less than 10 mg. This is important, for in certain investigations it is desirable to use small amounts of a carcinogen since large doses may mask the effect of solvent, sex, age, or other factors. Shear and Leiter (*5*) found that small amounts were necessary to detect the influence of sex in susceptibility to the production of subcutaneous tumors in strain A mice by 3,4-benzpyrene. In their work the males showed a susceptibility 50 percent greater than that of the females.

Induction of pulmonary tumors by the 10-mg. dose and hemangio-endotheliomas by the 20-mg. dose shows that the earlier 100-mg. amount was not essential for the production of these tumors and indicates that relatively small doses of the dye are capable of eliciting these neoplasms in tissues remote from the site of application.

From the data presented in the tables it may be concluded that there is a correlation between the amount of carcinogen administered and the tumor response, but the number of mice used was too small to determine accurately any dose-response relationship.

Law (*6*) and Morosenskaya (*7*) also administered 10 mg. of *o*-aminoazotoluene subcutaneously to mice and obtained tumors. Law dissolved the compound in olive oil and injected 5 mg. in three doses over a period of 2 months; a 5-mg. pellet was then inoculated; a total of 10 mg. was thus given to each animal. Hepatomas and fibrosarcomas at the injection site were induced. In the experiment reported here, fibrosarcomas were not induced by the crystalline compound, but in another in-

vestigation (*8*) the injection of an olive-oil solution of the compound produced this type of tumor. The difference between the present findings and Law's results may be attributed to his use of an olive-oil solution before the inoculation of the pellet. This is suggested by the appearance of a fibrosarcoma in one of his mice before the pellet was inserted. Morosenskaya dissolved the compound in sunflower-seed oil and gave four injections of 0.25 cc. each at 10-day intervals, or a total of 10 mg. Hepatomas and pulmonary tumors were induced.

The findings of Law and Morosenskaya, together with the results of the experiment reported in the present paper, show that a variety of tumors can be induced in mice by subcutaneous injection of 10 mg. of *o*-aminoazotoluene.

SUMMARY

Strain A female mice received subcutaneous injections of crystalline *o*-aminoazotoluene moistened with glycerol in graduated doses of 10, 20, 30, 40, 50, or 60 mg.

The 10-mg. dose induced hepatic lesions, hepatomas, and pulmonary tumors. The 20-, 30-, 40-, 50-, and 60-mg. doses induced hepatic lesions, hepatomas, pulmonary tumors, and hemangio-endotheliomas. There was a correlation between the amount of carcinogen administered and the induction of hepatic lesions and of the various tumors.

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Influence of Hybridization Upon the Occurrence of Mammary Tumors in Mice

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During the course of an experiment (*1*) involving reciprocal foster nursing between high- and low-mammary-cancer-strain mice, it was found that foster nursing of low-tumor strains C57 Black and I by high-tumor strain C3H produced an increase in the incidence of mammary tumors in the fostered mice, but hybrids derived by mating C57 Black females to strain I males and foster nursed by strain C3H showed a higher incidence of mammary tumors than did either of the parental strains. These results suggested that the cross-breeding of two low-tumor strains produced hybrids which were more susceptible to the development of mammary carcinomas than were their parents. The results were not considered conclusive since the fostered C57 Black mice and C57 Black × I hybrids were not offspring of the same C57 Black mothers, and it was conceivable that the mothers of the hybrid animals were more susceptible than the mothers of the C57 Blacks. In the experiment reported here this possibility was eliminated by using C57 Blacks and C57 Black × I hybrids that were born to the same C57 Black mothers.

The experiment is one of a series carried out during the past few years to determine the characteristics of various inbred strains of mice and their hybrids so that it may be known which test animal is most desirable for certain problems in experimental cancer.

MATERIALS AND METHODS

Mice of strains C3H, C57 Black, and I were used. The C3H animals were from a subline of the strain maintained at the National Cancer Institute (*2*). The C57 Black mice were descendants of a litter obtained from Dr. H. J. Bagg, of the Memorial Hospital, New York. This litter was from his substrain of C57 Black mice (*3*) in which no mammary tumors occurred under the "functional test" method of breeding. The substrain has undergone 7 generations of inbreeding in this laboratory, and in 350 breeding females the mammary-tumor incidence is less than 1 percent. The strain I mice were descendants of a litter supplied by the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine, in 1935. This strain has undergone 15 generations of inbreeding in this laboratory, and in 1,100 breeding females the incidence of mammary cancer is less than 1 percent.

The experiment was begun during February 1940 by brother-to-sister matings of strain C57 Black for their first litters. As the young were born, half the females of each litter were given to a strain C3H foster mother. After their first litters were weaned, the C57 Black females were mated to strain I males, and half the females of each resultant litter were transferred to a strain C3H foster mother. This procedure gave two groups of animals,

both derived from the same C57 Black females. The first group consisted of strain C57 Black mice nursed by strain C3H foster mothers and litter-mate controls nursed by their C57 Black mothers, and the second group consisted of C57 Black × I hybrids nursed by strain C3H foster mothers and litter-mate controls, nursed by their C57 Black mothers. To ascertain the susceptibility of strain I females to the mammary-tumor inciter in strain C3H milk, new-born strain I mice were given to strain C3H foster mothers.

The mice were weaned when they were 1 month old, and 1 month later each was mated and permitted to bear three litters in rapid succession; each litter was killed within 24 hours after birth. According to Bagg (*3*), rapid breeding and non-suckling increase the incidence of mammary cancers in mice. The animals were kept under similar environmental conditions and were given an unlimited supply of Purina dog chow and drinking water.

The diagnosis of mammary tumor was made by microscopic examination.

RESULTS

The results of the experiment, summarized in table 1, confirm the previous finding (*1*) in that F_1 hybrids derived from strains C57 Black and I were more susceptible to the mammary-tumor inciter in strain C3H milk than was either parental strain.

A 63-percent tumor incidence in fostered C57 Black mice is high for this strain and is comparable with the findings of De Ome (*4*) who used high-mammary-cancer-strain A females as foster mothers for his C57 Black animals and obtained a tumor incidence of 20.9 percent at an average age of 13.9 months. De Ome indicated that an increase in the incidence was expected because 76, or 58.9 percent, of the fostered C57 Blacks were alive at an average age of 12.6 months, which was lower than the mean age of 13.9 months at which tumors had occurred in the positive mice.

De Ome's animals were offspring of mice procured from the Jackson Memorial Laboratory. As stated previously, the ancestors of the C57 Black mice used in the present experiment were obtained from Dr. Bagg, who also procured his original stock from the Jackson Memorial Laboratory. Since De Ome's mice and those used here were descendants of animals obtained from the same laboratory, it is possible that they were members of the same C57 Black substrain which may account for the similarity of results in the fostered mice.

If the mice were related, it is evident that they were resistant to the development of mammary tumors when nursed by their mothers, for De Ome did not find any such neoplasms in 61 breeding females that lived to an average age of 15.9 months, and in this laboratory their mammary tumor incidence is less than 1 percent. Therefore, the high incidence of tumors produced when the mice were foster nursed by females of strains A or C3H is additional evidence that the foster nursing of low-mammary-tumor-strain mice by high-mammary-tumor-strain females produces a definite increase in the incidence of tumors in the fostered mice.

Table 1 shows that a mammary tumor occurred in 1 of 25 unfostered C57 Black mice. This tumor is of special interest, for it emphasizes the fact that this neoplasm occurs in greater or less degree in all strains of mice. The mother of the mouse in which the tumor arose bore 5 litters and died without tumor at the age of 13 months. Mammary tumor has not been found in any of her ancestors or descendants in the colony. The tumor-bearing mouse had 5 female litter mates; 2 were fostered by a strain C3H female and

TABLE 1.—*Occurrence of mammary tumors in female mice of strain C 57 Black and in strain C57 Black × I hybrids nursed by their mothers or strain C3H females, and in females of strain I nursed by strain C3H females*

[Numerator=mice developing tumors
Denominator=mice dying without tumors]

Strain of mother	Strain of father	Nursed by strain—	Number of mice	Time in months																			Total	Incidence of mammary tumors	Average tumor age	Average age of mice dying without tumor
				5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23				
																								Pct.	*Months*	*Months*
C57 Black	C57 Black	C3H	35						1/0	1/0		4/1	5/0	4/1	2/0	2/1	2/0	1/2	0/3	0/1	0/2	0/1	22/13	63	14.7	19.5
Do	do	C57 Black	25							0/1					0/3	0/2		1/3	0/2	0/2			1/24	4	19.0	19.1
Do	I	C3H	41	2/0	5/0	3/0	2/0	5/0	6/0	2/0	3/2	2/0	1/6			1/0		1/1	0/3	0/2			33/8	80	9.6	18.1
Do	I	C57 Black	40										0/2	0/5	0/1					0/14			0/40	0		19.3
I	I	C3H	43			0/1	0/1		0/8	0/3	0/1	1/6	0/4	0/15	0/3				0/18				1/42	2	13.0	13.0

developed mammary cancers when 13.5 and 19.5 months of age; 3 served as unfostered controls and died tumor-free at the ages of 15.5, 18.5, and 22 months, respectively.

Data on the incidence of mammary carcinomas in the fostered C57 Black × I hybrids and their litter-mate controls are given in table 1. The influence of hybridization upon susceptibility to tumor growth will be discussed later.

Only 1 of 43 fostered strain I females developed a mammary tumor; the rest died at an average age of 13 months. Mice of strain I die at a relatively early age because of the occurrence of rectal prolapse or an adenomatous stomach lesion (*5*). Table 1 shows that the average age at death of the fostered I mice was higher than the average age at which the C57 Black × I hybrids developed tumor. Thus, the I mice were more resistant to the C3H milk agent than were the C57 Black × I hybrids.

The incidence of mammary tumors in the mothers and foster mothers of the experimental mice was analyzed to ascertain whether their susceptibility could have influenced the outcome of the investigation. Twenty-six strain C57 Black females were used to procure the experimental C57 Black animals and the C57 Black × I hybrids, and none developed a mammary cancer. Death occurred when the mice were between the ages of 13 and 24.6 months, the average age at death being 17.5 months. The number of litters born to each mouse varied from 2 to 7, the average being 4.5. Twenty-one strain C3H females were used to foster nurse the C57 Black young, and 18 (86 percent) developed mammary tumors at an average age of 7.8 months; 3 died without tumor at 6.5, 7, and 7 months of age, respectively. Nineteen strain C3H females served as foster mothers for the C57 Black × I hybrids, and 17 (90 percent) developed mammary tumors at an average age of 8.1 months; 1 died when 8 months old. The 15 strain C3H females which foster nursed the strain I mice developed mammary tumors at an average age of 7.2 months. The fostered I mice were the offspring of 28 I females, none of which developed a mammary tumor; their average age at death was 12 months. These incidences are considered normal in mice of the 3 strains, and it is clear that the results in the experimental mice cannot be attributed to a variation in the degree of susceptibility of their mothers or foster mothers.

In view of Bittner's observation (*6*) that the incidence of mammary tumors in the first and second litters born to each mother is less than that for the following litters, the data were analyzed to ascertain whether this factor was responsible for the difference in tumor incidence between the C57 Black and the C57 Black × I hybrids. The incidence of tumors and the mean tumor age were determined according to number of the litter that had been born to each strain C3H female before she was used as a foster mother. The data are summarized in table 2. None of the C57 Black mice were fostered by C3H females bearing their first litter, and the differences in incidence between the C57 Black animals and the C57 Black × I hybrids that were fostered by C3H females bearing their second, third, or fourth litter are not significant. However, the hybrids fostered by C3H females bearing their fourth litter developed tumors at an average age of 7.5 months, whereas the average age of the other three groups varied from 10 to 11 months. The number of mice is too small to confirm or disprove Bittner's findings, but his observation concerning the correlation between tumor incidence and successive litters demands an analysis of all

TABLE 2.—*Incidence of mammary tumors in fostered C57 Black mice and in C57 Black × I hybrids according to the number of litters born to the foster mothers*

Number of litter when C3H female was used for foster nursing	C57 Black				C57 Black × I hybrids			
	Number of mice	Tumor incidence	Average tumor age	Average age of mice dying without tumor	Number of mice	Tumor incidence	Average tumor age	Average age of mice dying without tumor
		Percent	*Months*	*Months*		*Percent*	*Months*	*Months*
1					8	88	10	12
2	15	60	15.5	19.5	9	78	10.7	16.5
3	16	63	13.6	19.2	10	80	11	20.5
4	4	75	16	21	14	79	7.5	19.7

foster-nursing results upon the basis of the number of litters born to the foster mothers. Since the fostered C57 Black mice represented the first litters and the C57 Black × I hybrids the second litters born to the C57 Black females, the difference in incidences was not ascribable to the number of litters cast by the C57 Black females. Bittner's observations could be interpreted on the basis of an intra-uterine influence as well as the milk influence.

DISCUSSION

Studies on the influence of hybridization upon the susceptibility of mice to various types of malignancies have been in progress for several years. These studies have shown that when highly inbred strains of known susceptibility are crossed it is not possible to predict the degree of susceptibility of the resultant hybrids. Hybridization (*7*, *8*) of strains C3H and I gave offspring that were more susceptible to induced pulmonary tumors than their parents, whereas hybridization of strains C3H and Y produced offspring that were at least as resistant as the more resistant parent C3H strain. Outcrossing of strain C3H to strains Y or I produced hybrids that were of intermediate susceptibility to induced subcutaneous tumors when compared with their parents. Hybrids (*9*) procured from strains I and L were tested for susceptibility to progressive growth of transplantable sarcoma 37, and 87 percent were susceptible although virtually all mice of strains I and L were resistant.

Studies (*7*, *8*) on the inheritance of susceptibility to mammary tumors revealed that the breeding of strain C3H females to strain I males produced offspring of high susceptibility, but the breeding of strain C3H females to males of strains Y or C57 Black resulted in offspring that were more resistant than the more susceptible C3H parent strain.

In the experiment reported here hybrids derived from strain C57 Black females and strain I males were more susceptible to the development of mammary tumors than either parental strain. Hence, they resembled the C3H × I hybrids in susceptibility to induced pulmonary tumors and the I × L hybrids in susceptibility to progressive growth of sarcoma 37.

Geneticists recognize that the hybrid mean may exceed the parental mean. Wright (*10*) outcrossed inbred guinea pigs and tested the hybrids for susceptibility to tuberculosis. Some were more resistant than either parental family. Russell (*11*, *p. 340*) stated: "The average of a character in the F_1 may fall between the parental averages, it may correspond to either parent, or it may lie beyond the parental value." Gordon (*12*) found that the mating of a nontumorous spotted platyfish (*Platyspoecilus maculatus*) with a nontumorous Mexican swordtail (*Xiphophorus*

hellerii) produced hybrids that developed melanotic neoplasms. Little (*13, p. 270*) crossed two species of mice, *Mus bactrianus* and the C57 Black strain *M. musculus*, and obtained a striking increase in the incidence of tumors in the hybrids. Both Gordon's and Little's results were from species crosses, while those presented here were from inbred strains within a species. Korteweg (*14*) also drew attention to the influence of hybridization upon the susceptibility of mice to spontaneous mammary carcinoma.

The precise manner in which hybridization affects the occurrence of mammary tumors in mice is obscure. It is known (*15*) that at least three influences are essential for the development of this neoplasm: (1) An agent which is usually transmitted through the milk of the mother but which is widely distributed throughout the body; (2) the hormonal influence; and (3) the genetic influence. The genetic influence determines the degree of susceptibility to the agent or the hormonal influence or both. Hybridization affects the genetic influence and may, therefore, increase or decrease the susceptibility of the animals to either or both of the other influences.

Regardless of the role played by the genetic influence in the production of mammary tumor in the mouse, the present findings again emphasize the importance of hybridization in the occurrence of this neoplasm. In previous experiments (*16*), high-tumor-strain females were mated to low-tumor-strain males, and the female hybrids thus obtained were also mated to low-tumor-strain males; the resultant backcross mice developed few mammary cancers. This breeding procedure permitted passage of the milk influence but concentrated the genetic influence of the low-tumor strains to such a degree that the backcross mice were resistant to mammary tumors. As shown here, only one generation of breeding was necessary to obtain susceptible mice from two relatively resistant strains.

Such observations on the effect of hybridization upon the development of mammary tumors in mice may have some bearing upon the influence of heredity in the occurrence of tumors in other species. Outcrossing strains of mice of known genetic uniformity and ancestry may result in offspring which exceed the parental values. This indicates that in other species which do not approach the genetic uniformity of inbred mice it is difficult to predict the occurrence of tumors on the basis of tumorous or nontumorous ancestry.

SUMMARY

Female mice of low-mammary-tumor-strain C57 Black were mated to their brothers, and half the females of each litter were foster nursed by high-mammary-tumor-strain C3H females. Sixty-three percent of the fostered mice developed mammary cancers at an average age of 14.7 months, whereas 4 percent of the nonfostered litter mates did so at an average age of 19 months. The C57 Black females were next mated to low-mammary-cancer-strain I males, and half the females of each litter were foster nursed by strain C3H females. Eighty percent of the fostered mice developed tumors at an average age of 9.6 months, whereas none of the litter-mate controls did so.

Strain I females were foster nursed by strain C3H females, and 2 percent developed tumors at an average age of 11 months.

The results indicate that hybrids derived from strains C57 Black and I are more susceptible to the development of mammary cancers than is either of the parental strains.

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Absorption of Subcutaneously Implanted Pellets of Diethylstilbestrol in Men

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It appears to be established that estrogenation (*1*, *2*) or castration (*3*, *4*) are effective measures in the palliative treatment of inoperable prostatic carcinoma. The use of diethylstilbestrol or diethylstilbestrol dipropionate has some advantages over castration, particularly in the ease of administration, the avoidance of a major surgical procedure, and the low cost. Moreover, approximately one-fourth of the patients with prostatic carcinoma do not respond to castration (*3*, *4*), and in these cases estrogenation can be tried as a supplemental procedure.

The optimum dosage of stilbestrol in prostatic carcinoma has not been established, but the beneficial response is obtained by an average dose of 1 to 3 mg. per day. Kahle et al. (*1*) gave intramuscular injections in oil, 5 mg. two or three times a week, and no toxic reactions were encountered when this dose was given for as long as 17 months. The compounds are also effective by the oral route.

The need for constant, protracted ingestion or injection of stilbestrol in prostatic carcinoma suggested the administration of the compounds in the form of subcutaneously implanted pellets. MacBryde et al. (*5*) reported that the average daily absorption of pellets of diethylstilbestrol dipropionate weighing 100 mg. is 0.127 to 0.25 mg. for the first 50 days of observation. This method of administration in women was stated to be 5 to 10 times more effective per unit weight than the oral or the usual parenteral route. The slow rate of absorption of diethylstilbestrol dipropionate, and the slower rates of absorption and the prohibitive cost of α-estradiol and α-estradiol benzoate (*6*) indicated the use of diethylstilbestrol, which has been shown to be absorbed more rapidly in rats than the above-mentioned compounds (*7*).

Nine male patients with neoplastic disease were implanted subcutaneously with sterilized pellets of diethylstilbestrol.[1] The pellets, made by compression, were cylindrical, 3.2 mm. in diameter and 7.5 to 8.0 mm. long, and weighed 49.0 to 53.0 mg. The average surface area of the pellets was 96 mm.^{2} and the volume 64 mm.^{3}, so that 0.78 mg. was compressed into each cubic millimeter.

The subcutaneous implantations were made through a paracentetic trocar, after a small incision had been made through the skin in the seventh intercostal space at the posterior axillary line. The pellets were implanted at least 2 cm. away from the incision. The skin incision was approximated with sterile tape and healed within 48 hours. At intervals following the implantation, the pellets were removed, dried, and weighed in order to determine the absorption rate, as presented in table 1 and figure 1.

None of the pellets was extruded or produced a local reaction other than a thin, fibrous, but well-vascularized capsule that did not interfere with the absorption of the material. None of the patients

[1] *We are indebted to Dr. Edwin Schwenk, of the Schering Corporation, Bloomfield, N. J., for furnishing these pellets.*

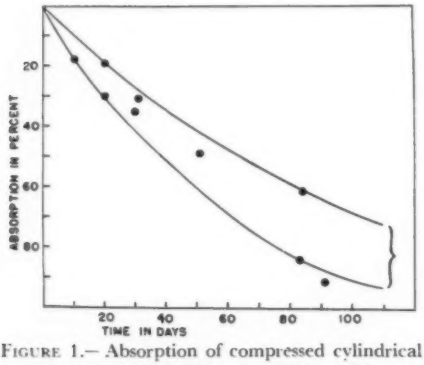

FIGURE 1.— Absorption of compressed cylindrical pellets of diethylstilbestrol weighing 50 mg., implanted subcutaneously in men.

had local discomfort during the administration of the pellets or during the time the pellets remained *in situ*. Inspection of the figure shows that during the first 50 days, 40 to 60 percent of the weight was absorbed from the 50-mg. pellets and that in 100 days 70 to 90 percent of the original weight was absorbed.

Thus, one 50-mg. pellet of the compression and shape used delivered an average of 0.5 mg. per day for 50 days and an average of 0.4 mg. per day during a 100-day period.

The nine patients were also implanted with two compressed pellets of diethylstilbestrol weighing 25 mg. and containing 50 percent of cholesterol.[2] The absorption of these pellets in 100 days was 20 to 40 percent, thus yielding an average of 0.05 to 0.10 mg. of stilbestrol per day. However, 3 of 10 pellets removed more than 50 days after implantation were found to be partially or completely disintegrated. Such pellets are not recommended for clinical use.

All four patients that received one 50-mg. pellet of diethylstilbestrol and two 25-mg. pellets of stilbestrol with 50 percent cholesterol and in whom such pellets remained for 50 days or longer (yielding an average of 0.475 to 0.75 mg. of diethylstilbestrol per day), developed some enlargement of the breasts, and in two the breasts became tender. No toxic manifestations such as nausea developed. Obvious beneficial effects as far as the malignant condition was concerned were evident only in the patient with prostatic carcinoma with metastases to the spine and pelvis.

Pellets of diethylstilbestrol[3] weighing 50 mg. and measuring 2.7 mm. in diameter and 8 to 9 mm. in length, and pellets of diethylstilbestrol containing 10 or 25 percent of cholesterol, some of the same weight and size and others weighing approximately 70 mg. and measuring 2.7 mm. in diameter and 12 mm. in length, were also prepared by fusion (*8*). This method of preparing pellets consists of melting the hormone, with or without

[2] *See footnote 1.*

[3] *We are indebted to Merck & Co., Rahway, N. J., for the supply of diethylstilbestrol.*

TABLE 1.—*Absorption of compressed cylindrical pellets of diethylstilbestrol implanted subcutaneously in men*

Patient	Diagnosis	Age	Original weight of pellet	Time implanted	Final weight of pellet
		Years	*Milligrams*	*Days*	*Milligrams*
W. O	Carcinoma, stomach	52	49.2	10	40.2
J. W	Melanoma, generalized	77	51.0	20	41.4
J. H	Sarcoma, thigh	51	51.6	20	35.8
J. F	Carcinoma, larynx	74	52.5	30	34.3
C. G	do	68	53.0	31	36.4
W. P	Carcinoma, lung	54	49.0	51	25.1
L. A	Carcinoma, stomach	63	49.6	83	8.0
A. H	do	64	49.0	84	19.0
F. J	Carcinoma, prostate	47	51.5	91	4.9

cholesterol, in an oil bath and slowly drawing the liquid into a lightly oiled glass or metal tube by means of suction produced by a well-fitting plunger. The hormone solidifies quickly in the form of a solid string which is extruded by the plunger. The solid string is cut with a razor blade into sections of desired size. The resultant pellets are washed in alcohol and packed into test tubes for additional sterilization in the autoclave.

Pellets produced in this manner are as hard as those formed by compression; in fact, in the present series the compressed pellets contained 0.78 mg. of material per cubic millimeter, whereas the fused pellets contained 1.1 mg. of hormone in the same volume. The melting point of diethylstilbestrol is 169°–171° C. (corrected), so that the maintenance of this temperature for 15 minutes before the preparation of the pellets assures the destruction of pathogenic organisms and spores (*9*).[4]

[4] *It is felt that the pouring of molten hormones into molds of desired shape and size may be a simple and effective method of preparing sterile hormone pellets for subcutaneous implantation. The combination of cholesterol makes the preparation of diethylstilbestrol pellets much simpler, as the pellets have less tendency to split upon cooling, but is not applicable to some other hormones, such as testosterone propionate, as such mixtures are eutectic.*

Since the absorption of subcutaneously implanted pellets is primarily dependent upon the surface area, disk-shaped pellets are absorbed in a more uniform manner than are cylindrical pellets (*7*). The latter shape is preferable, however, from the standpoint of ease of administration, since an ordinary paracentetic trocar and a minimal skin incision are needed for the introduction of cylindrical pellets of the size used.

Fused diethylstilbestrol pellets, with or without cholesterol, have been implanted into 17 men with neoplastic disease, including 4 patients with carcinoma of the prostate. The men received 2 to 4 pellets each, or approximately 100 to 200 mg. of stilbestrol. Data on absorption are available to date on 7 patients and are presented in table 2.

Pellets containing 10 percent of cholesterol absorbed at a rate of approximately 50 percent, and pellets containing 25 percent of cholesterol absorbed at a rate of approximately 30 percent in 100 days. The pellets remained *in situ* without disintegration and produced little if any more reaction than compressed pellets of diethylstilbestrol without cholesterol. Further observations are necessary, however, before stilbestrol-cholesterol pellets can be

TABLE 2.—*Absorption of subcutaneously implanted cylindrical pellets of diethylstilbestrol made by fusion*

Patient	Diagnosis	Age	Cholesterol	Original weight of pellet	Time implanted	Final weight of pellet
		Years	*Percent*	*Milligrams*	*Days*	*Milligrams*
J. G.	Carcinoma, tongue	59	10	69.5	2	68.0
			25	69.4	2	68.1
D. W.	Carcinoma, nasopharynx	54	10	69.7	28	48.3
			25	69.7	28	54.0
C. M.	Carcinoma, bladder	59	0	54.5	30	35.0
			10	50.1	30	38.4
E. L.	do	50	0	55.5	47	38.5
			10	54.9	47	43.2
A. H.	Carcinoma, lung	45	10	69.7	62	47.9
			25	68.7	62	55.9
J. R.	Carcinoma, stomach	47	10	69.8	91	40.5
			25	70.9	91	63.2
J. W.	Hodgkin's disease	40	10	70.0	131	22.1
			25	70.0	131	37.0

recommended clinically, although the method is efficient in reducing the absorption rate of this and other steroid hormones (*8*) The fused pellets containing no cholesterol absorbed at approximately the same rate as the pellets made by compression.

Enlargement of the breasts was a constant response in 5 to 8 weeks after the implantation of the pellets. No toxic reactions were encountered. The period of observation is insufficient to evaluate the effects of the therapy on neoplastic disease. In two patients with carcinoma of the prostate, one had definite relief from pain within 2 weeks and in the other the elevated serum "acid" phosphatase (*10*) dropped 40 percent in 5 days and 90 percent in 40 days following the implantation of pellets.

CONCLUSION

The subcutaneous implantation of 50-mg. cylindrical pellets of diethylstilbestrol, which yielded a constant average daily absorption of 0.35 to 0.45 mg. for at least 100 days, seems to be an efficient and desirable method of administration of stilbestrol to man. Sterile pellets can be prepared by fusion. The implantation of two or three pellets of stilbestrol, yielding a daily dose of 0.8 to 1.2 mg., appears to be effective in the palliative treatment of carcinoma of the prostate.

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Types of Tumor Induced by Ultraviolet Radiation and Factors Influencing Their Relative Incidence

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Ultraviolet radiation penetrates the skin of mice to some depth and reaches a variety of tissue elements upon which it may act (*1*). Hence it might be anticipated that a variety of types of tumor would be induced by this agent. The most superficial layers receive more of the incident energy and would be expected to be the most common site of tumors, but the findings herein reported indicate that other factors play important, if not predominant, roles in determining the types of tumors induced. The pathologic studies described in the present paper were made on tumors resulting from a variety of dosages of ultraviolet radiation, the quantitative aspects of which have been treated elsewhere (*2, 3*).

PATHOLOGIC FINDINGS

In a previous paper (*4*), which was based on a series of 144 tumors, the histologic features of the predominant tumor types were discussed. The most common type of tumor encountered was fibrosarcoma derived from the subepidermal tissues. Less commonly noted was squamous carcinoma arising in the epidermis or its appendages. We have now examined tumors of the ears of 605 strain A mice subjected to a wide range of dosages and intensities of ultraviolet radiation. As in the previous series spindle-cell sarcomas originating in the subepidermal tissues have predominated. Many, though by no means all, of these growths have been stained with Mallory's phosphotungstic acid-haematoxylin solution, and either Foot's or Wilder's stain for reticulum. On the basis of the presence of typical fibroglia fibrils and the amount and distribution of reticulum most of these tumors have been classified as fibrosarcomas. In a relatively small number of spindle-cell tumors the occurrence of myosarcoma has been considered, particularly in connection with those spindle-cell sarcomas which were composed of more plump and fleshy cells than are usually noted and in which bizarre giant cells were common constituents. We have never observed cross striations in undoubted tumor cells or even convincing evidence of longitudinal striation. In the absence of these characteristics the occurrence of myoglia fibrils, as described by Mallory in sections stained according to his phosphotungstic acid method, has been adopted as the criterion of myosarcoma. In two tumors, cells possessing thick, blue-staining fibrils springing from the extremities of the cells and frequently terminating in hooklets were seen. It is quite probable that these tumors were of muscular origin and it is also probable that more tumors of this type would have been diagnosed if the technique had been used on all. However, the total number certainly is not large in comparison with those sarcomas of obvious fibroblastic nature.

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In addition three types of tumor which did not occur in the first series have been recorded, namely, hemangio-endothelioma, osteochondrosarcoma and sebaceous carcinoma. The most common was hemangio-endothelioma, which developed in the ears of five mice. Grossly, these lesions were small, oval or round nodules of a striking wine-purple cast, covered by intact epidermis. Four of the five were composed of large cavernous spaces filled with erythrocytes and lined by flattened or swollen endothelial cells. The vascular spaces were tortuous and separated from each other by relatively thick bands of connective tissue. The lesions were well-circumscribed, and their histologic appearance did not suggest malignant properties. Figure 1 represents a section of one of these tumors. The fifth tumor was composed of large numbers of vascular slits or poorly formed capillaries lined by swollen endothelial cells and containing few erythrocytes. The vessels were closely packed and separated by thin strips of fibrous tissue which were infiltrated by granulocytes. These characteristics are illustrated in figure 2. While considerably less differentiated than the others, this tumor could not well be considered malignant. The rather frequent occurrence of hemangio-endotheliomas of the eye as well as of the ear indicated that these tumors were not spontaneous but were induced by the action of ultraviolet radiation.

In one animal a tumor arising in the ear as a firm, reddish, crusted growth $8 \times 6 \times 5$ mm. had the microscopic character of an osteochondrosarcoma. There were nests of large, closely packed, mononuclear tumor cells of obviously cartilaginous character and several large irregular masses of osseous tissue including areas of endochondral ossification, as shown in figure 3. The tumor was large and locally invasive but did not give rise to metastases. Associated with this tumor was a small squamous carcinoma. This osteochondrosarcoma was the only example of such a tumor, but proliferative lesions of the aural cartilage plate resulting in local nodules, such as illustrated in figure 4, or ectopic cartilaginous islands were frequently observed. Thus, it seems probable that this single tumor was the result of the action of ultraviolet radiation rather than a tumor of spontaneous origin.

The third tumor, the only example of its type, was a crusted oval mass 9 x 7 x 5 mm., microscopically made up of broad sheets of large mononuclear cells with abundant, finely vacuolated cytoplasm. This is illustrated in figure 5. Fine droplets of sudanophilic material were demonstrated in the cytoplasm. There was relatively little collagenous stroma, and the reticulum was sparse and widely separated. In view of the close resemblance to cells of the sebaceous glands, the presence of stainable lipoid, and the stromal pattern, this tumor was classified as a sebaceous carcinoma. Abnormal proliferation of sebaceous gland cells has been commonly observed in the ears of animals exposed to ultraviolet radiation, which led us to believe that the tumor resulted from the action of this agent. The significance of tumors arising from sebaceous glands, cartilage, and vascular endothelium is perhaps best explained in terms of penetration of ultraviolet radiation. Appreciable quantities of such radiation penetrate the epidermis of the mouse ear and are absorbed in the dermal tissue (*1*), and this may account in part for the high proportion of sarcomatous growths observed in mouse skin as contrasted with the prevalence of epidermal growths in human skin. For the same reason, the occurrence of tumors derived from sebaceous glands,

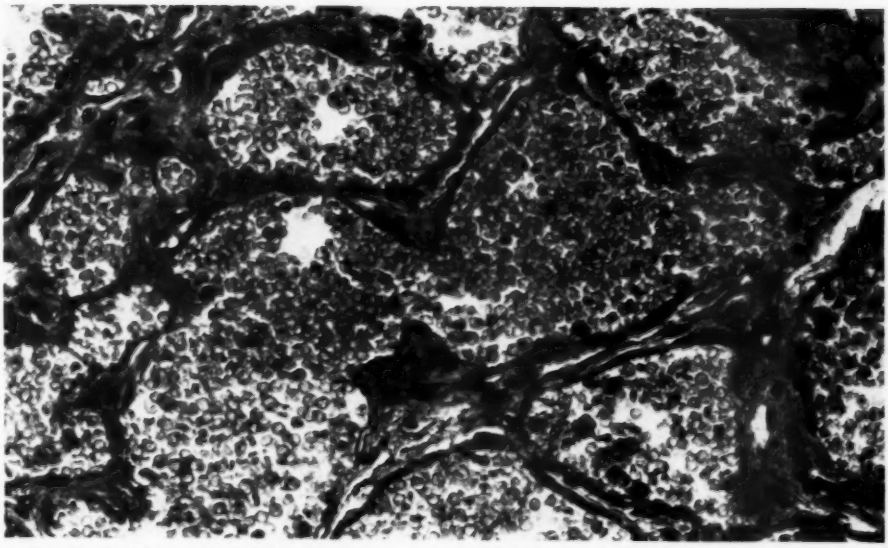

FIGURE 1.—Hemangio-endothelioma, cavernous type. Haematoxylin and eosin stain. × 200.

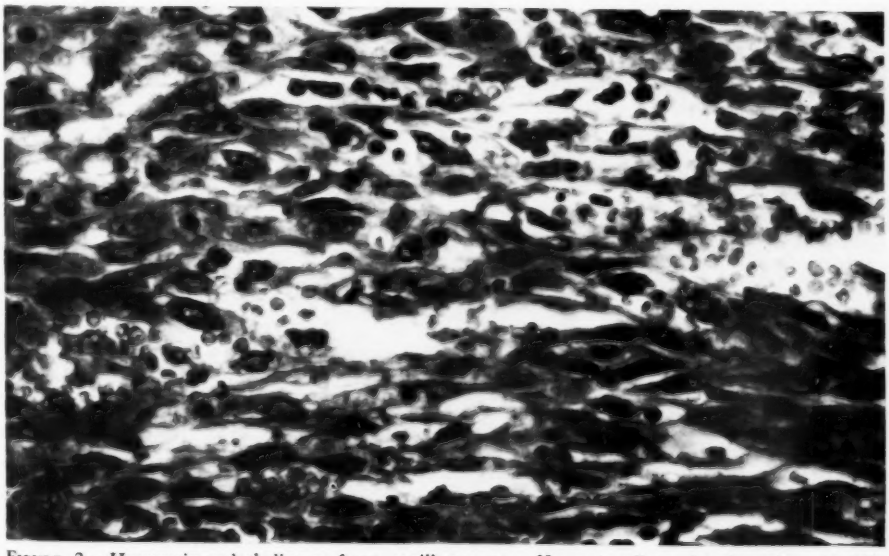

FIGURE 2.—Hemangio-endothelioma of ear, capillary type. Haematoxylin and eosin stain. × 285.

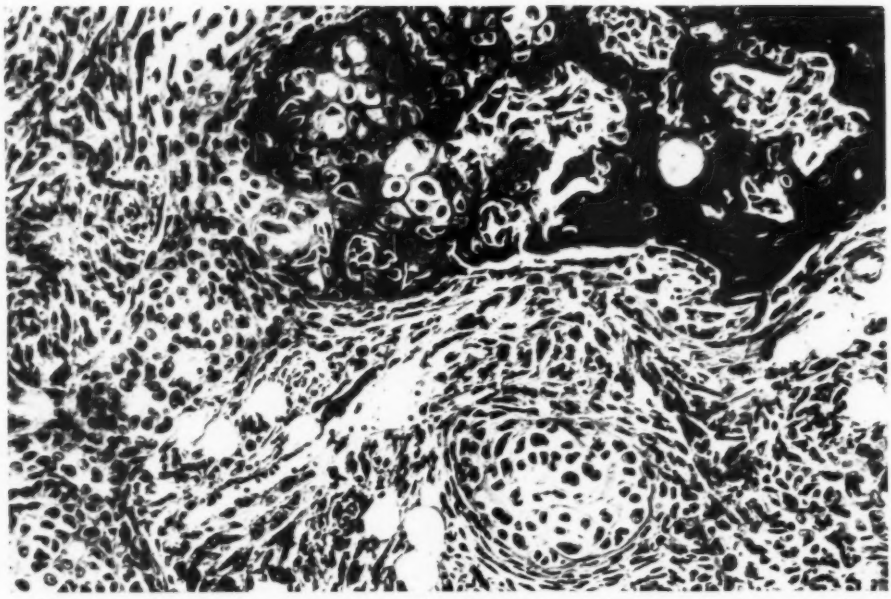

FIGURE 3.—Osteochondrosarcoma of ear. Phosphotungstic acid-haematoxylin stain. × 200.

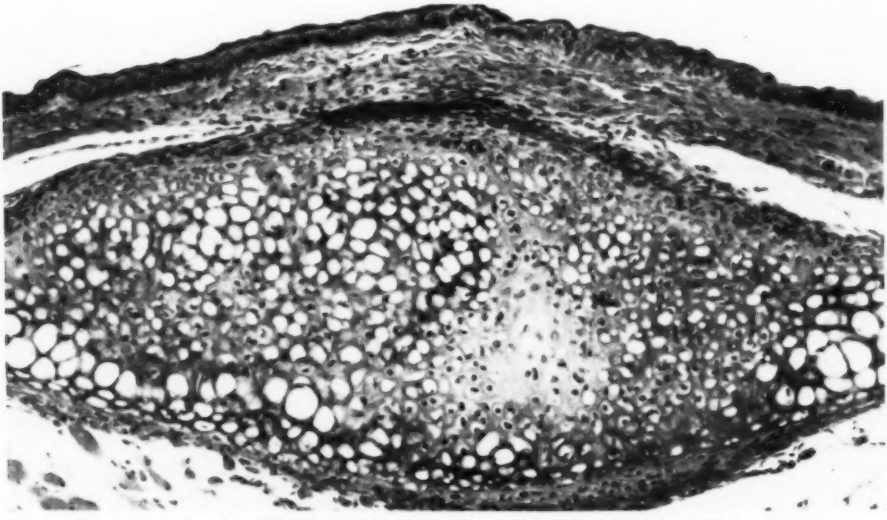

FIGURE 4.—Local proliferation of cartilage of ear. Note thickening of perichondrium. Haematoxylin and eosin stain. × 200.

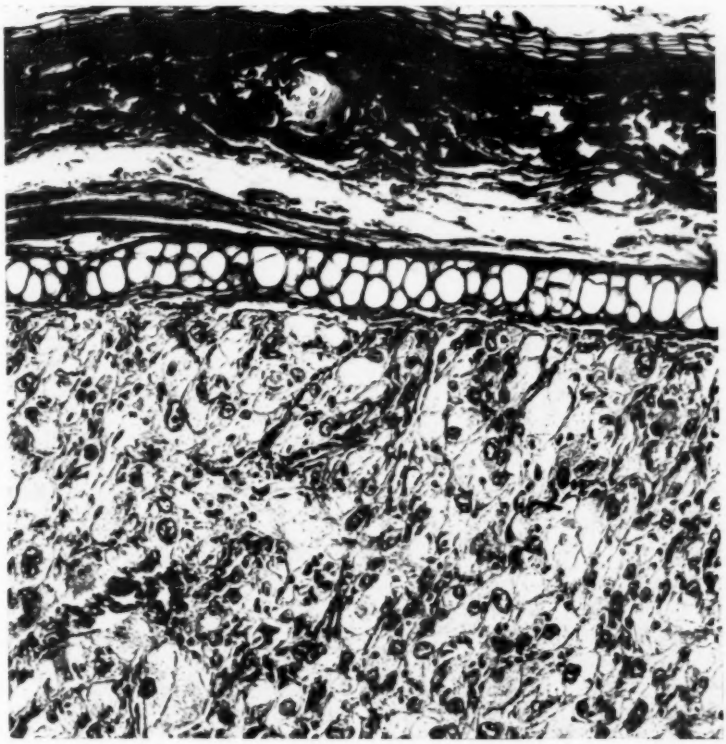

FIGURE 5.—Sebaceous carcinoma of ear. Note similarity of tumor cells to sebaceous gland cell at top of figure. Phosphotungstic acid-haematoxylin stain. ×200.

cartilage, and vascular endothelium is not surprising since the radiation reached these tissues.

In this connection the primary tumors arising in the eye are of interest. Primary tumors occurred in the eyes of about 5 percent of the mice. They have not been included in the statistical analyses because of their infrequency and irregular distribution. Most of the tumors of the eye arose in the cornea, particularly the substantia propria. A few were large growths widely invading the orbital tissues, and because of their extensive nature it has been impossible to determine their point of origin. Grossly, they appeared first as flat, opaque growths obscuring the pupil and later protruding through the lids as dry, crusted, or hemorrhagic masses. A few of them which began as films on the cornea had a characteristic port-wine color. Microscopically, the tumors were of two types, spindle-cell sarcomas or fibrosarcomas, and hemangio-endotheliomas. The smaller sarcomas were confined to the substantia propria of the cornea and frequently were covered externally by hyperplastic corneal epithelium, although this was often ulcerated. They also extended laterally and invaded sclera and choroid. It seems reasonable in view of the constancy of location of the smaller growths to assume that many of the large tumors also arose in the cornea. They

were composed of bundles of long spindle-shaped cells with hyperchromatic nuclei and fibrillar cytoplasm essentially similar histologically to those observed in the dermis of the ear. The occurrence of, and pattern of reticulum fibrils were also typical of fibrosarcoma. Not uncommonly large numbers of granulocytes invaded the tumors in which the corneal epithelium had ulcerated. In view of local invasive properties and cellular morphology, these lesions were considered malignant although metastases were not found.

The hemangio-endotheliomas which were frequently diagnosed on gross examination by their striking port-wine color also arose in the substantia propria of the cornea and in most instances were confined to that location. Large, cavernous, blood-filled spaces lined by single or occasionally multiple layers of endothelial cells honeycombed the substantia propria. Small strips of fibrous tissue separated the vessels and not infrequently contained scattered macrophages filled with hemosiderin. These tumors were almost invariably of similar pattern and showed little tendency to the morphologic variations noted by Andervont, Grady, and Edwards (*5*) in hemangio-endotheliomas induced by *o*-aminoazotoluene. We have not seen any wide invasion by tumors of this type although they were not allowed to reach maximum size. Nothing in the growth rate, aggressive tendencies, or microscopic qualities so far observed would permit an unqualified diagnosis of malignancy, particularly since the behavior of hemangio-endothelioma cannot always be correlated accurately with histologic appearance (*6*).

Considered as a whole, the evidence indicates that ultraviolet radiation may induce tumors in any susceptible tissue it reaches. Depth of penetration of the radiation is obviously a limiting factor, which has been discussed elsewhere (*1*, *4*, *7*), but difference in response of the tissues to this agent may also be a factor in determining the relative incidence of tumor types. The following analysis of the numerical distribution of tumor types clearly demonstrates such a difference.

DISTRIBUTION OF TUMOR TYPES

The tumor diagnoses were distributed as follows among the 605 mice from which tumors of the ear were examined histologically: Spindle-cell or polymorphous-cell sarcomas were found unaccompanied by other tumors in 72 percent. Carcinomas of the epidermis or its appendages were present unaccompanied in 5 percent, while such tumors were present together with spindle-cell or polymorphous-cell sarcomas in 22 percent. The remaining types of tumor (hemangio-endothelioma, osteochondrosarcoma, sebaceous carcinoma) constituted about 1 percent.

The last group is so small that it has been neglected in analysis of the data. Examination of the ratio of carcinomas to sarcomas among the remaining animals reveals interesting relationships. In this analysis carcinomas and sarcomas have been considered as constituting two separate groups of tumors, regardless of whether they are present alone or accompanied by other tumors. No distinction is made between fibrosarcomas and possible myosarcomas, since the diagnosis of the latter is somewhat uncertain; and since they are few in number, no important error can be introduced by including them in the group.

In table 1 carcinoma–sarcoma ratios are shown for different dosage groups, all of which received five exposures per week. This material was obtained from the groups discussed previously from other aspects (*2*, *3*). All the material available

for each dose has been used regardless of the intensity of the radiation. The table shows clearly that there is no correlation between dose of radiation *per se*, and the carcinoma–sarcoma ratio.

TABLE 1.—*Distribution of tumor types with respect to dose*

Weekly dose (ergs/square centimeter)	Total number of animals	Tumor type		
		Carcinomas	Sarcomas	Ratio Carcinomas/sarcomas
		Number	*Number*	
43.0	15	5	15	0.333
16.5	62	9	59	.153
13.0	95	27	90	.300
9.9	77	12	75	.160
7.9	70	20	67	.298
5.3	66	20	62	.323
3.6	76	19	72	.264

It was thought possible that carcinomas might grow somewhat more slowly than sarcomas, without reference to dosage, and hence that the carcinoma-sarcoma ratio would be somewhat higher among individuals in which the tumors appeared later, i. e., those with longer individual induction times. To test this hypothesis, the data in table 1 were broken up into groups according to individual induction time, without respect to dose (table 2). There is a trend toward increase in the carcinoma–sarcoma ratio with increase in induction time which suggests a difference in rate of growth of the two types of tumor. The trend might conceivably be explained on the basis of age of the animals.

The ratio of carcinomas to sarcomas is thus very nearly, if not completely, independent of dosage of radiation per se, of age of the animals, and of over-all rate of growth of the tumors.

In contrast, the ratio is markedly affected by the interval between exposures, as is shown in table 3. If the mice are exposed to the radiation only 1 day per week, the carcinoma–sarcoma ratio is much lower than if they are exposed every day of the week, the 5-day-per-week schedule of exposures being represented by an intermediate carcinoma–sarcoma ratio.

TABLE 2.—*Distribution of tumor types with respect to individual induction time*

Individual induction time (in days)	Total number of animals	Tumor type		
		Carcinoma	Sarcoma	Ratio Carcinoma/sarcoma
		Number	*Number*	
100–150	112	20	106	0.189
150–200	186	45	177	.254
200–250	88	23	86	.267
250–300	42	12	40	.300
300+	33	12	31	.387

TABLE 3.—*Distribution of tumor types with respect to schedule of exposures*

Number of exposures per week	Total number of animals	Tumor type		
		Carcinoma	Sarcoma	Ratio Carcinoma/sarcoma
		Number	*Number*	
1	60	3	60	0.050
5	461	112	440	.255
7	77	32	68	.470

The variation in the ratios shown in table 3 demonstrates clearly a difference in response of epidermal and connective tissue elements. This difference cannot be explained on the basis of different energy thresholds since the ratio is not correlated with dose (table 1). For the same reason it cannot be explained on the basis of tissue damage, hyperplasia, or screening action of the epidermis (*3*), inasmuch as these factors vary widely with dose. Since the two types of tissue respond differently with respect to the interval between doses, a temporal factor of some kind must enter. Several plausible explanations might be offered, but prolonged discussion does not seem justified without additional evidence.

SUMMARY

The histopathology of tumors induced by a wide variety of dosages of ultraviolet radiation was studied. Spindle-cell sarcomas obviously arising from connective tissue elements predominated. A few polymorphous-cell sarcomas occurred which possibly originated from muscle cells. Squamous carcinomas arising in the epidermis or its appendages formed a smaller though important group, often occurring with sarcomas. A few hemangio-endotheliomas, one osteochondrosarcoma, and one sebaceous carcinoma were also found.

Hemangio-endotheliomas and spindle-cell sarcomas of the eye are described.

It is concluded that ultraviolet radiation may induce tumors in a variety of tissues depending upon their susceptibilities and limited by the penetration of the radiation.

The ratio of carcinomas to sarcomas was examined. Dose per se did not affect the ratio, which might have increased slightly, however, with induction time. On the other hand, interval between exposures had a marked effect, the ratio increasing with frequency of exposure. This demonstrates a difference in response of epidermal and connective tissue elements.

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Role of Thrombocytopenia in Hemorrhage Produced in Sarcoma 37

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The injection of filtrate of cultures of *Bacillus prodigiosus*, *B. coli*, or certain other bacteria into mice bearing sarcoma 37 or certain other tumors produces hemorrhage in the neoplasm.[1] It is believed that bacterial filtrates exert their action by damaging the tumor blood vessels with resultant bleeding and necrosis in the tumor (*2*).

Two factors at least may be involved in the production of this type of hemorrhage: the damage to the endothelium of the blood vessels and the removal of the platelets from the circulation (*3, 4*). Tocantins[2] noted that injection of *Bacillus prodigiosus* filtrate into rabbits resulted in a drop in the platelet count of the peripheral blood to as low as 92,000 per cubic millimeter. The bleeding time, leukocyte count, and red-blood-cell count were not markedly affected. Anaphylactic shock, which was shown by Barrett (*5*) to produce hemorrhage in sarcoma 37, also is accompanied by a marked drop in platelets (*6*). These findings suggested the investigation of the role which thrombocytopenia plays in hemorrhage produced in sarcoma 37 by bacterial products and other agents.

EXPERIMENTAL PROCEDURE

The test animals were strain A backcross mice, approximately 4 months old and weighing 26 to 30 gm. Sarcoma 37, which is maintained by subcutaneous passage in strain dba mice, was minced and inoculated into the shaved skin of the abdomen of the animals (*7*). Six or seven days after the grafting of the tumors, the masses were readily visible, and the mice received the test materials.

The injections were made intravenously into the lateral tail vein or intraperitoneally. In all instances the material was dissolved in 0.2 cc. of distilled water, and only a single injection was given.

Blood for counting the platelets was obtained by cutting off the peripheral segments of the tail. Mixed venous and arterial blood was thus secured. The platelets were counted after dilution in Tocantins' modification of Rees-Ecker fluid (*8*).

EXPERIMENT 1

A lyophilized concentrate of a filtrate of *Bacillus prodigiosus*[3] was dissolved in sufficient distilled water to contain 0.01 mg. per cubic centimeter.

It was determined that 0.002 mg. of the material dissolved in 0.2 cc. of water and given as a single intravenous injection produced hemorrhage in more than 90 percent of the tumors within 6 hours after the administration (table 1). With this dose, the platelet count dropped from the normal 500,000 to 900,000 to as low as 200,000 within 6 hours (fig. 1). There was no significant drop in the red-blood-cell count.

Gross and histologic examination of the skin and visceral organs of the animals, killed at various intervals after injection, failed to reveal hemorrhages in the kidneys, intestines, or lungs. The tumors showed

[1] *For review of literature, see Shear* (1) *and Andervont and Shimkin* (2).

[2] *Tocantins, L. M.: Personal communication (1942).*

[3] *This sample, prepared in 1940 by M. J. Shear and F. C. Turner* (9) *and lyophilized by J. White, of the National Cancer Institute, was known as K8dl.*

extravasation of blood and, at later stages, necrosis and inflammation in the areas involved by hemorrhage.

TABLE 1.—*Effect of bacterial filtrate concentrate, anti-mouse-platelet serum, and of moccasin venom on strain A backcross mice implanted with sarcoma 37*

Material	Concentration per cubic centimeter	Dose	Mice injected	Mice with hemorrhage in tumor	Mice with petechiae on toes, ears, etc.
	Milligrams	*Cc.*	*Number*	*Number*	*Number*
Bacterial filtrate concentrate.	0.01	0.2	14	13	0
		.1	11	8	0
		.05	10	6	0
		.025	15	4	0
		.0125	8	0	0
Anti-mouse platelet serum.		.3	5	3	5
		.2	14	5	14
		.2	16	1	7
		.05	19	0	0
		.025	7	0	0
Moccasin venom	1.0	.1	17	9	5
		.05	5	2	0
		.025	5	0	0
		.0125	5	0	0

EXPERIMENT 2

Bedson (*3, 4*) has described the immunologic properties of antiplatelet serum. The preparation of this material by the method of Bull and McKee (*10*) permitted an evaluation of the role of the thrombocytopenia produced by the bacterial filtrate.

Approximately 400 mice provided 200 cc. of oxalated mouse blood which was obtained by cutting off the forearm. Washed platelets were separated from red and white cells by means of differential centrifugation, and the platelets were lyophilized. Intraperitoneal injections of lyophilized platelets from 50 cc. of mouse blood were given to a guinea pig four times at weekly intervals. Six days following the last injection the guinea pig was bled and the serum removed and lyophilized.

The lyophilized anti-mouse-platelet serum was dissolved in distilled water in amounts equal to the original volume of serum unless higher concentrations were needed. Graded doses were injected intravenously into mice bearing sarcoma 37. Platelet counts were made before and at intervals following the injection.

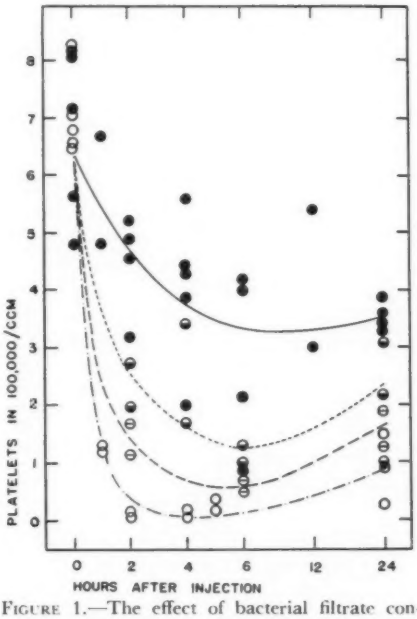

FIGURE 1.—The effect of bacterial filtrate concentrate and of anti-mouse-platelet serum on the number of platelets in peripheral blood in mice.

Solid line and solid points, 0.2 cc. of bacterial filtrate concentrate. Short dash line and half-open points, 0.05 cc. of antiplatelet serum. Long dash line and lined open points, 0.1 cc. of antiplatelet serum. Dot-dash line and open points, 0.2 cc. of antiplatelet serum.

As shown in figure 1, the drop in the number of platelets was directly related to the dose of the serum. With 0.2 cc. of serum, the platelet count dropped to less than 50,000 and most of the animals died within 24 hours. There was a uniform appearance of petechial hemorrhages on the toes, the clipped ears, and in the intestines and lungs.

With the higher doses of the antiplatelet serum, hemorrhages also appeared in the

tumors (table 1), most markedly in tumors that were traumatized by the insertion of the animals into a bleeding holder. The extent of the hemorrhages, however, did not approach that obtained with bacterial filtrate. There was no correlation between the amount of hemorrhagic extravasation into the tumors and the number and extent of petechial hemorrhages in other tissues.

When 0.05 cc. of the antiplatelet serum was administered, the platelet count dropped to less than 200,000 within 4 hours (fig. 1). Despite the drop in the platelet count to the same level as that produced by the bacterial filtrate, the mice did not develop hemorrhages in the transplanted tumors (table 1). It was evident that thrombocytopenia was not primarily the cause of hemorrhage in sarcoma 37 elicited by bacterial filtrate.

Twenty-one mice bearing sarcoma 37 received a single intravenous injection of 0.05 cc. of bacterial filtrate mixed with 0.05 cc. of the antiplatelet serum; the total volume was adjusted to 0.2 cc. Twelve mice developed hemorrhage in the tumor. Since with 0.05 cc. of the filtrate alone 6 of 10 mice developed hemorrhage in the tumor, it was concluded that the action of the bacterial filtrate was not enhanced by the additional reduction in platelets produced by the antiplatelet serum.

Experiment 3

Similar experiments were performed with lyophilized moccasin (*Agkistrodon piscivorus*) venom.[4] The material was dissolved in distilled water to equal 1 mg. per cubic centimeter and was injected intraperitoneally.

It was found that 0.2 cc. of the material killed the mice. When 0.1 cc. was used, 9 of 17 mice bearing sarcoma 37 developed hemorrhage in the tumor. The hemorrhage in some instances was as extensive as that obtained with bacterial filtrate concentrate. Five of seventeen animals also developed petechial hemorrhages on the toes and clipped ears. The platelet count with this dose dropped to as low as 80,000 per cubic millimeter within 6 hours after injection; in 24 hours the platelet count was 290,000 and 350,000.

Ten mice received a combination of 0.5 cc. of anti-mouse-platelet serum and 0.1 cc. of moccasin venom. Four animals developed hemorrhage in the tumor. The number and the extent of the hemorrhages in the tumors were, therefore, not enhanced by the addition of antiplatelet serum to the snake venom.

DISCUSSION

The results show that the injection of two substances that produce hemorrhage in transplanted sarcoma 37, a concentrate of a filtrate of *Bacillus prodigiosus* and moccasin venom, also produces a definite drop in the number of platelets in the peripheral blood.

Experiments with anti-mouse-platelet serum indicate that the hemorrhages in the tumors are not the result of the thrombocytopenia produced. The reduction of the platelets to similar or lower levels than those obtained with bacterial filtrate did not induce hemorrhages in the tumors. The results are in harmony with the work of Bedson (*3, 4*), who found that antiplatelet serum alone did not produce capillary hemorrhage, but that it was necessary to add some capillary toxic agent before extravasation of blood occurred. The hemorrhages that occur in thrombocytopenic

[4] *Obtained from the Ross Allen Reptile Institute, Silver Springs, Fla.*

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animals are caused by exogenous or endogenous injury to the capillaries which is not rapidly repaired.

The fact that simple reduction of the platelets does not produce hemorrhages in the tumors strongly suggests that a capillary toxic factor of the bacterial filtrate and the moccasin venom is the active agent in the production of hemorrhages in the tumors, and that the drop in platelets is incidental. Rich and Follis (*11*) showed that the endothelium is injured in the Arthus reaction. The injury to the endothelium suggests that the mechanism of the hemorrhages obtained by anaphylactic shock is also ascribable to direct capillary injury.

With doses of antiplatelet serum sufficient to produce consistent petechial extravasation of blood in the skin, intestine, and lung, very few hemorrhages were elicited in sarcoma 37. On the other hand, with doses of bacterial filtrate sufficient to cause marked hemorrhage in the sarcoma, no petechial hemorrhages were present in the normal skin or the visceral organs. These results suggest that the capillaries of sarcoma 37 are more susceptible to certain injurious agents than are the normal capillaries.

CONCLUSION

Thrombocytopenia produced by anti-mouse-platelet serum, of the same level as that produced by a concentrate of *Bacillus prodigiosus* filtrate and by moccasin venom, did not produce hemorrhage in transplanted sarcoma 37 in strain A backcross mice. The action of the bacterial filtrate and of the snake venom on the transplanted sarcoma 37 is primarily that of an endothelial toxin.

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Hydrolysis of Glutathione by the Cathepsins of Normal Rat Tissues and Rat Hepatoma 31

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INTRODUCTION

The ability of the endocellular proteinases or cathepsins to hydrolyze glutathione is of special interest because of the activating power which this naturally occurring tripeptide exerts upon many biologically important enzymes as well as upon the cathepsins themselves. This hydrolytic action of the cathepsins on glutathione may represent a reversal of the process by which glutathione is synthesized since the endocellular function of these enzymes is probably both synthetic and hydrolytic.

In early experiments designed to determine the structure of glutathione, Grassmann, Dyckerhoff, and Eibeler (*1*) found that glutathione was not attacked by pepsin, pancreas proteinase, or papain. In remarkable contrast with other synthetic peptides which had been studied, they found that glutathione also resisted the actions of the dipeptidases and the aminopolypeptidases of yeast and of the intestines. They found that the carboxypolypeptidase of hog pancreas readily hydrolyzed glycine from the disulfide form, and they were able to isolate 80 percent of the glycine liberated. The sulfhydryl form was not hydrolyzed. Abderhalden and Geidel (*2*) did not obtain catheptic hydrolysis of glutathione and several of its derivatives when they used a glycerine extract of hog liver which had been dried with alcohol and ether as the source of cathepsin.

Woodward, Munro, and Schroeder (*3, 4*) found that kidney and pancreas extracts of various species contained an enzyme which hydrolyzed glutathione at an optimum pH of 7.0. They named this enzyme antiglyoxalase because it inhibited glyoxalase by hydrolyzing its activator, glutathione.

In an earlier paper (*5*) we compared the peptidase activities of the cathepsins of normal rat tissue and of Jensen rat sarcoma, using as substrates various dipeptides and polypeptides including glutathione. Recently hepatoma 31, a transplantable tumor of known hepatic origin, became available in quantities sufficient for the separation of the catheptic fraction and afforded material for a valid comparison of the rates and mode of action of the proteolytic enzymes of a neoplastic tissue and its normal tissue of origin.

EXPERIMENTAL METHODS

Enzyme Preparations

The catheptic preparations were separated by the procedure previously described (*6*) from the following tissues: liver, kidney, and spleen from normal rats, the livers of rats resistant to hepatoma 31 as shown by regression or the failure of the tumors to grow when transplanted, and hepatoma 31 from subcutaneous transplants, originally induced by feeding *p*-dimethylaminoazobenzene (*7*) to Osborne-Mendel strain rats. Tests showed that these cathepsin preparations did not pos-

sess catalase or arginase activity, or dipeptidase and aminopolypeptidase activity at an alkaline pH. These dried enzyme preparations were water-soluble and contained from 12 to 14 percent total nitrogen and from 7 to 8 percent protein nitrogen precipitable by 0.3M trichloracetic acid.

The digests contained 15.35 mg. (0.05 mm.) of reduced glutathione per cubic centimeter of citrate buffer at pH 5.0. The resulting pH was 4.5. For the pH activity determinations both citrate and phosphate buffers were used. The anaerobic digests were incubated in evacuated Thunberg tubes and were kept at 40° C. in a water bath.

The degree of hydrolysis of the glutathione was determined by titration of the carboxyl groups in alcohol with alcoholic N/40 KOH by using thymolphthalein as indicator according to the technique of Grassmann and Heyde (*8*). In some experiments the Harris HCl method (*9*) was also used, but this procedure was complicated by the presence of the buffers in the digests.

In other experiments the titrations were checked by determinations of the carboxyl carbon according to the manometric technique of Van Slyke, Dillon, Mac Fadyen, and Hamilton (*10*). The amount of cysteine liberated by the hydrolysis was determined by the method of Sullivan and Hess (*11*).

RESULTS

The normal liver and hepatoma cathepsins exhibited a marked optimum at pH 4.5 and only slight activity in the alkaline pH range (fig. 1). This peptidase activity was therefore catheptic, i. e., related to the endocellular proteinases and not due to contaminating peptidases with optima in the alkaline pH range. Whether the proteinase and peptidase activities are caused by separate enzymes or are dual functions of one catheptic enzyme has not been determined.

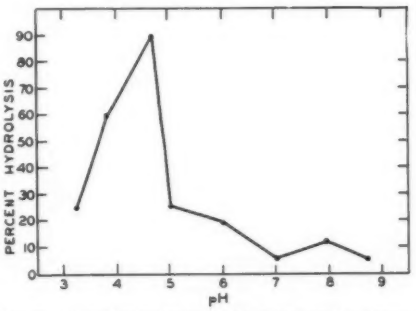

FIGURE 1.—Hydrolysis of glutathione by hepatoma 31 cathepsin in 4 hours.

The catheptic preparations from all of the rat tissues tested were able to hydrolyze the glycine linkage of the glutathione molecule. The order in which the peptide bonds were attacked was proved by the isolation of γ-glutamylcysteine and glycine (as hippuric acid), from the digests when the hydrolysis had proceeded until the titration with alkali indicated that the equivalent of one carboxyl group had been liberated. The negative cysteine test at this point was proof that the two peptide linkages were not attacked simultaneously. A digest containing 460.50 mg. glutathione in solution in 24 cc. water and 60 mg. of hepatoma cathepsin dissolved in 6 cc. citrate buffer at pH 5.0 was incubated at 40° anaerobically for 18 hours. The titration then showed that 89 percent of one carboxyl group had been liberated, and the test for cysteine was negative. From this digest 102 mg. of γ-glutamylcysteine was separated by mercury and copper precipitations which had the following composition:

Carboxyl carbon	N *Percent*
Found, 5.1 mg. substance 0.245 mg. C. . .	10.9
Theory, 5.1 mg. substance 0.245 mg. C. . .	11.2

The substance melted with sintering at 163° C. Harington and Mead (*12*) found that synthetic γ-glutamylcysteine melted at 167°. The glycine was separated by benzoylation, and 82 mg. of hippuric acid was obtained from the filtrate of the mercuric sulphate precipitation of the γ-glutamylcysteine. Poor yields were obtained in these separations because of the number of necessary manipulations, but separation of 27 percent of the γ-glutamylcysteine and 30 percent of the glycine afforded supporting evidence for the titration values and the negative test for cysteine. The results obtained with the normal liver cathepsin and glutathione digests were similar to those obtained with the cathepsin from hepatoma 31 after an 18-hour hydrolysis in which the titration showed that the equivalent of one carboxyl group had been liberated.

The rate at which the normal liver cathepsin hydrolyzed the glycine bond was about twice as great as the rate for the hepatoma cathepsin, judging from the K values for the hydrolysis of this bond (table 1). However, the hepatoma cathepsin hydrolyzed the second peptide bond after 22.5 hours of hydrolysis while the normal liver cathepsin did not hydrolyze this bond even after 36 hours, with the purposely large proportions of enzyme to substrate which were used in these experiments. By using a smaller quantity of hepatoma cathepsin (1 mg.) at a more acid pH of 3.5, 28 percent of the γ-glutamylcysteine was hydrolyzed in 40 hours. This difference in ability to hydrolyze the

TABLE 1.—*Hydrolysis of glutathione by cathepsins from rat tissues and from hepatoma 31*

Source of enzyme	Concentration of enzyme	Anaerobic hydrolysis of glutathione					
		pH	Time	By titration [1]	K [2]	Cysteine liberated [3]	Carboxyl carbon [1]
	Milligrams per cubic centimeter		*Minutes*	*Percent*		*Percent*	*Percent*
Normal rat liver	2	4.5	20	35	0.02155	0	
			40	57	.02106	0	
			60	72	.02121	0	
			180	72		0	
			1,430	86		0	85
			2,200	86		0	84
Hepatoma 31	2	4.5	22	20.4	.01013	0	
			40	34.0	.01038	0	
			90	61.3	.01049	0	
			300	86.3		0	
			1,430	123		29	122
			2,200	152		50.2	156
Do	1	3.5	30	16.5	.005994	0	
			60	30.0	.005938	0	
			120	51.0	.005941	0	
			1,200	100.0		0	
			2,400	128.0		26.4	
Livers of rats resistant to hepatoma 31	1	4.5	30	32.6	.01313	0	
			60	55.9	.01364	0	
			120	78.2	.01268	0	
			1,200	78.0		0	
Normal rat spleen	2	4.5	30	48	.02179		
			60	86			
			330	96			
			1,200	133		39	142
			2,400	189		92	197
Normal rat kidney	1	4.5	30	16	.005799	0	
			60	30	.005938	0	
			120	50	.005776	0	
			1,430	85		0	
			2,400	90		? trace	

[1] The hydrolysis equivalent to 1 carboxyl group = 100 percent.

[2] $K = \frac{1}{T} \times 2.303 \log \frac{A}{A-X}$.

[3] These percentages are based on the total quantity of cysteine available by hydrolysis.

γ-glutamylcysteine suggests that the liver cathepsin may have been altered when the normal liver cells became neoplastic. No cysteine was found until the titration values and the increase in carboxyl carbon indicated that the equivalent of one peptide bond had been hydrolyzed. The amount of cysteine liberated by the hepatoma and spleen enzymes corrresponded very closely to the degree of hydrolysis of the γ-glutamyl-cysteine bond as determined by the increase in carboxyl carbon and by the titration of liberated carboxyl groups.

This ability to hydrolyze both peptide linkages of the glutathione was not limited to the hepatoma cathepsin but was also shown by the normal spleen cathepsin and to a greater degree. Since the K values were not significant after about 75 percent of the glycine bond had been hydrolyzed, only one K value could be given for the spleen cathepsin. The rate of hydrolysis for the γ-glutamyl-cysteine bond is much slower than for the γ-glutamylcysteine-glycine bond, both with the spleen cathepsin and the hepatoma cathepsin. This may be related to the more general inability of most catheptic enzymes to attack dipeptides as shown previously (*5*).

The rate of hydrolysis of glutathione by the normal kidney cathepsin was similar to that of the hepatoma cathepsin at pH 3.5 and was about half as great as the rate for normal liver cathepsin, or that for the cathepsin from livers of hepatoma-resistant rats. The comparative decrease of the ability of the kidney cathepsin to hydrolyze glutathione differentiates this enzyme from the antiglyoxalase of Woodward, Munro, and Schroeder which was most potent in kidney extracts.

The cathepsin prepared from the livers of resistant rats showed a very slightly higher rate of hydrolysis than that of the cathepsin prepared from normal livers when equal amounts of enzyme were used.

SUMMARY

Cathepsins prepared from normal rat livers, livers of rats resistant to hepatoma 31, normal spleen and kidney, and hepatoma 31 hydrolyzed the glycine bond of glutathione at an optimum pH of 4.5.

The order in which the peptide bonds of glutathione were attacked was proved by the absence of free cysteine and the separation of γ-glutamylcysteine and glycine from the digests when the alkalimetric titration indicated that the equivalent of one peptide bond had been hydrolyzed.

The cathepsin from normal rat livers hydrolyzed the glycine peptide bond of glutathione at twice as great a rate as that of the hepatoma cathepsin. But the hepatoma cathepsin was able to hydrolyze slowly the remaining γ-glutamylcysteine while the normal liver cathepsin did not attack this dipeptide. The normal spleen cathepsin also hydrolyzed both bonds of glutathione and at a much more rapid rate than did the hepatoma cathepsin. The rate of hydrolysis of glutathione by the cathepsin from livers of resistant rats was very slightly higher than the cathepsin from normal rat livers. The rate of the kidney cathepsin was about one-half that of the normal liver cathepsin.

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Cytochrome Oxidase and *d*-Amino Acid Oxidase in Tumor Tissue

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The metabolism of tumors under both aerobic and anaerobic conditions has been extensively investigated in recent years. It is generally agreed that tumors derive a considerable portion of their energy requirements from fermentative rather than oxidative processes, in spite of the substantial respiration exhibited by many tumors. The elucidation of the chemical basis for this phenomenon requires detailed knowledge of the individual enzymes and substrates concerned with the utilization of energy as well as of the overall metabolism. In this paper are reported measurements of two enzyme systems which react directly with oxygen, cytochrome oxidase and *d*-amino acid oxidase. Although a variety of tumor and normal tissues has been studied, particular attention is given to tissues of hepatic origin. Several transplanted hepatomas available in this Institute are compared with normal liver, liver of tumor-bearing animals, liver regenerating after partial hepatectomy, and fetal liver. The work reported in this paper supplements previous enzyme studies on these homologous tissues (*1–4*).

CYTOCHROME OXIDASE

It is now believed that the major portion of cellular oxidations does not involve direct union of oxygen with metabolites but proceeds through a series of carriers consisting of iron catalysts, flavoproteins, pyridine nucleotides, dehydrogenases, and perhaps others. The first link in the main chain is the cytochrome system, a group of iron porphyrin proteins which function by undergoing a reversible ferro-ferri change. The present knowledge of these systems has recently been summarized by Stotz (*5*). To date five components have been identified: cytochrome oxidase, and cytochromes *a*, a_3, *b*, and *c*.[1] Of these only cytochrome *c* has been isolated and purified. Study of the others, which have never been separated from insoluble cell fragments, has largely depended on their sharp spectral absorption in the reduced state and specific inhibition by such compounds as KCN, H_2S, and azide. According to current opinion, cytochrome oxidase is oxidized by molecular oxygen and reduced by the ferro-cytochromes, the ferri-cytochromes being in turn reduced by specific flavoproteins. Whether cytochromes *a*, *b*, and *c* function in parallel or in series in this oxidative chain is still an open question.

MATERIALS AND METHOD

Osborne-Mendel and Buffalo rats and mice of strains C3H, A, I × dilute brown, and dba were used in the experiment. Most of the animals were fed an unrestricted diet of Purina dog chow.

The method employed for determination of the oxidase is similar to the methods described by Keilin and Hartee (*7*) and Stotz (*8*). It involves the measurement of the oxygen consumption of ground tissue suspensions in the presence of an excess of cytochrome *c* and of a substrate reacting directly with cytochrome *c*, in this case *p*-phenylenediamine. Under these conditions the oxidase is the limiting factor in

[1] *For discussion concerning possible identity of the oxidase and* a_3, *see Keilin and Hartree* (6).

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the chain of reactions involving O_2, cytochrome oxidase, cytochrome *c*, and *p*-phenylenediamine.

The oxygen consumption was measured manometrically by the Warburg technique. The flasks contained 1 cc. of 0.3 M phosphate buffer of pH 7.20, 1 cc. of pure cytochrome *c* solution[2] (1×10^{-7} moles per cubic centimeter), and 1 cc. of tissue suspension. The center cup contained 0.2 cc. of 20 percent KOH, and the side arm 0.3 cc. of freshly prepared *p*-phenylenediamine solution (6.6 mg.). After equilibration the *p*-phenylenediamine solution was mixed with the cell contents, and the manometers were read at 5-minute intervals for 20–30 minutes, during which the oxygen consumption was always linear with time. The temperature was 38° C., and the rate of shaking 120.

The hourly oxygen consumption was calculated from the 5-20 minute period. *p*-Phenylenediamine rather than hydroquinone was used as substrate to avoid the high rate of autoxidation of the latter, which also varies markedly with pH. Although *p*-phenylenediamine, unlike hydroquinone, also reacts with O_2 via the autoxidizable cytochrome *b* (*10*), a correction was made by measurement of O_2 consumption as indicated previously, but with the cytochrome oxidase poisoned by KCN substituted for the added cytochrome *c*. This blank, only a few percent of the total oxygen uptake, represents oxidation of *p*-phenylenediamine (or substrates in the tissue) through autoxidizable, cyanide-insensitive compounds such as cytochrome *b*. It was in all cases subtracted from the total respiration, the recorded $Q_{O_2}^{ox}$ being

$$Q_{O_2}^{ox} = \frac{\text{Total } O_2 \text{ per hour} - O_2 \text{ per hour with cyanide}}{\text{Milligrams}}$$

The rats were killed by decapitation, the mice by breaking their necks. The tissues were immediately removed, the necrotic portion of the tumors was dissected out as far as possible, and weighed samples (100 to 600 mg.) were ground into 20 ml. of 0.1 M phosphate buffer of pH 7.2. In some cases, the homogenizing apparatus described by Potter and Elvehjem was used; in others the tissue was ground with a little sand in a mortar. The bulk of the sand settled rapidly, and the tissue suspension could then be poured off. No significant differences in the results of the two methods were found.

All tissue suspensions were kept in an ice bath after the homogenization until used. Under these conditions, the oxidase was stable since the $Q_{O_2}^{ox}$ values were unchanged even after the suspension had stood several hours. In general, differences between samples of the same tissue or of different aliquots of the same homogenized material were not greater than 3 to 5 percent. This variation is well within the individual variation between animals, which was of the order of 10 to 20 percent. It was found, in agreement with Stotz (*8*) and Schultze (*11*), that the $Q_{O_2}^{ox}$ values were independent of the quantity of tissue used, provided that the oxygen consumption was less than 550–600 mm.[3] per hour.

Table 1 shows that the cytochrome oxidase activity of transplanted liver tumors is considerably lower than that of normal liver. In both rats and mice the ratio is about 3 to 5 whether figured on a wet- or a dry-weight basis. The various hepatomas differed only slightly, hardly more than might be expected in view of varying degrees of residual necrosis. No correlation between oxidase activity and tumor etiology, strain of animal or rate of tumor growth was found.

That the low value of tumor is not necessarily a characteristic of growing tissue is seen in the fact that regenerating liver, in

[2] *Prepared according to the method of Keilin and Hartree* (*9*).

TABLE 1.—*Cytochrome oxidase activity of liver and of hepatic tumors*

Tissue [1]	Primary tumors induced by—	$Q_{O_2}^{ox}$ (mm.³ per hr. per mg.)	
		Fresh tissue	Dry tissue
Osborne-Mendel rat:			
Normal liver		30.9	103
Hepatoma 31	*p*-Dimethylaminoazobenzene.	6.8	38
Regenerating liver (48 hours).		30.1	123
Liver of rat bearing hepatoma 31.		25.9	98
Fetal liver of rat		12.3	55
Necrotic tissue of hepatoma 31.		1.1	
Strain C3H mouse:			
Normal liver		27.1	87
Hepatoma 98/15	Spontaneous	4.8	20
Hepatoma E	2-Amino-5-azotoluene.	6.2	26
Strain A mouse:			
Normal liver		25.9	84
Hepatoma 587	Spontaneous	5.1	19.8
Hepatoma 7A/77	Carbon tetrachloride.	6.2	24.2
I × dilute brown mouse:			
Hepatoma 1	2-Amino-5-azotoluene.	6.3	26.8

[1] All of the tumors were transplants.

spite of its rapid growth, is almost identical with normal liver. This is in agreement with expectations from other enzyme studies on this pair of tissues and from the values of the various metabolic quotients. Fetal liver was intermediate in value between neoplastic and normal liver. However, owing to the presence in fetal liver of a large amount of blood-forming cells, it is impossible to state whether the cytochrome oxidase in fetal lever cells is actually lower or only diluted with non-hepatic cells.

A number of other tumors were studied. The results are given in table 2, which also refers to the results obtained by other investigators (indicated by italic numbers in parentheses). Values varied from 2.61 for Jensen sarcoma to 7.4 for leukosis Y 103. No correlation was found between these values and the tissue of origin, tumor etiology, strain of animal, or rate of tumor growth.

TABLE 2.—*Cytochrome oxidase activity of various tumor [1] tissues of the rat and the mouse*

Source of tissue	$Q_{O_2}^{ox}$ (mm.³ per hr. pr mg.)	
	Fresh tissue	Dry tissue
Osborne-Mendel rat:		
Hepatoma 31 (*12*)	6.8	38
Buffalo rat:		
Jensen sarcoma	2.61	10.2
C3H mouse:		
Hepatoma E (*13*)	6.2	26
Hepatoma 98/15 (*14*)	4.8	20
Adenocarcinoma of glandular portion of pyloric stomach (*15*)	3.0	11.0
Mammary adenocarcinoma	6.1	22.1
A mouse:		
Adenocarcinoma of small intestine (*16*)	4.4	20.8
Hepatoma 587 (*14*)	5.1	19.8
Hepatoma 7A/77 (*17*)	6.2	24.1
Leukosis 72,942 (*13*)	6.1	35.2
dba mouse:		
Leukosis Y 103 (*13*)	7.4	34.1
Malignant melanoma (*17*)	3.6	13.3
Sarcoma 37	6.3	23.0
Sarcoma CR 180	5.5	19.7
I × dilute brown mouse:		
Hepatoma 1 (*13*)	6.3	26.8

[1] All tumors were transplants, with the exception of the mammary adenocarcinoma.

Greenstein et al. (*1*) observed that the activity of liver catalase, an iron porphyrin enzyme, was markedly lowered by the presence of a tumor. To ascertain whether this was a generalized effect on iron porphyrins, various fresh tissues of normal Osborne-Mendel rats and of such rats bearing hepatoma 31 were compared with respect to cytochrome oxidase activity. The results, as shown in the following tabulation, show no significant differences from the values of the tissues of normal animals and indicate that there is no generalized systemic effect on iron porphyrin enzymes.

Tissue	$Q_{O_2}^{ox}$
Normal Osborne-Mendel rats:	
Liver	30.9
Spleen	9.5
Heart muscle	68.7
Skeletal muscle	8.2
Tumor-bearing Osborne-Mendel rats:	
Liver	25.9
Spleen	9.7
Heart muscle	69.1
Skeletal muscle	7.1

The tumors in table 2 were also examined for the presence of cytochromes *a* and *b* by the usual methods (*6*, *7*). After the tissue was ground in a meat grinder or minced with scissors, it was washed repeatedly with successive portions of distilled water until the supernatant was clear. The grinding of the tumor material into several volumes of M/15 KH_2PO_4 was followed by precipitation with an equal volume of acetate buffer of pH 4.5 and centrifugation. This last procedure was repeated until the hemoglobin bands disappeared from the supernatant. Then a thin suspension of the tissue in phosphate buffer was examined with a hand spectroscope after the suspension was reduced by the addition of $Na_2S_2O_4$. In all cases bands could be seen at 605 (α band of cytochrome *a*), at about 562 (α band of cytochrome *b*), and at 550 (α band of cytochrome *c*). Sometimes the weaker β bands of the cytochromes *a* and *c* (520 and 530) were also observed. Thus it appears that the complete cytochrome system was present in all the tumors examined.

d-AMINO ACID OXIDASE

Krebs (*19*) isolated from kidneys and liver an enzyme system which catalyzes the oxidative deamination of the *d* or unnatural amino acids to keto acids and ammonia. This enzyme, *d*-amino acid oxidase, was shown by Warburg and Christian (*20*) to be composed of a specific protein and a riboflavin-adenine-dinucleotide prosthetic group. Since the *d*-amino acid oxidase had been found in the livers of a variety of species, it was considered of interest to study its distribution in tumor tissues of hepatic origin.

The usual method for the determination of *d*-amino acid oxidase was employed (*19*). The tissue was either ground with sand or homogenized into three volumes of distilled water and extracted for 10 minutes at room temperature and then for 30 minutes to 1 hour at 0°. In some cases homogenized material was used, in others the supernatant after centrifugation. No differences in the results were found. Likewise, the activity remaining in the pellet was per unit volume equal to that in the supernatant. The increase in rate of consumption of oxygen or production of ammonia by the tissue extract on the addition of *d*,*l*-alanine was taken as a measure of the *d*-amino acid oxidase activity. Under these conditions the *l*-form is not attacked. Measurements were made by using the Warburg equipment. The vessels contained the following: 1 cc. of 0.1 M phosphate buffer, 2 cc. of extract representing 450–500 mg. of fresh tissue, 0.3 cc. of *d*,*l*-alanine solution containing 22 mg., and in the inner well 0.2 cc. of 20 percent KOH. In the control vessels 0.3 cc. of H_2O was substituted for the amino acid. After equilibration, the oxygen uptake was measured for 20 to 60 minutes. It was found that even though the total oxygen uptake gradually decreased in rate, the increment in rate owing to the addition of the amino acid was constant for at least 1 hour. Where ammonia determinations were desired, 1 cc. of 10 percent H_2SO_4 was added at the end of the reaction period to destroy the enzyme. Ammonia determinations were made by aeration into 0.2 percent H_2SO_4 followed by nesslerization.

RESULTS

The results are summarized in table 3. The averages represent the values obtained from at least six animals. Duplicates on the same sample of tissue agreed to within a few percent, but the variations among different animals were from 20 to 30 percent. Consequently, significance is at-

tached only to large differences between tissues. The riboflavin-adenine-dinucleotide content of each tissue is also given for reference.

TABLE 3.—d-*Amino acid oxidase activity in various tissues of the rat and mouse*

Tissue	Oxygen uptake ($mm.^3$ per hr. per mg.)	NH_3 ($mm.^3$ per hr. per mg.)	Flavin-adenine-dinucleotide per gram
		Fresh tissue	
			Micrograms
Osborne-Mendal rat:			
Normal liver	0.24	0.354	61
Liver of rat bearing hepatoma 31	.15		61
Regenerating liver	.19		52
Fetal liver	.035		11.5
Transplanted hepatoma 31	.022	.032	10.2
Kidney	1.61		60
Buffalo rat:			
Jensen sarcoma	0		7.5
Normal liver	.102		60
Liver of rat bearing Jensen sarcoma	.096		60
Strain A mouse:			
Normal liver	0	0	78
Liver of mouse bearing hepatoma	0		78
Transplanted hepatoma 587	0		32
Transplanted hepatoma 7A/-77	0		32
Strain C3H mouse:			
Normal liver	0	0	81
Liver of mouse bearing hepatoma	0		81
Transplanted hepatoma 98/-15	0		32
Kidney	.54		

The values of *d*-amino acid oxidase for normal liver and kidney of rats were about the same as those found for rats on an adequate diet by other investigators (*21*, *22*). Hepatoma 31 showed a low but definite activity, about 10 percent that of normal liver. Regenerating liver, in spite of its rapid rate of growth, was like normal liver rather than tumor. Although the average values for the livers of rats bearing hepatoma 31 was only 60 percent of that for normal liver, no great significance is attached to the difference inasmuch as there was some overlapping of the extreme values of both groups. Furthermore, in view of the dependence of *d*-amino acid oxidase activity on diet (*22*), such a small difference might well be owing to differences in food intake by normal and tumor-bearing animals rather than to a real systemic effect. The point can be settled only by accurately controlled dietary experiments. The value for fetal liver was low and was close to that for hepatoma 31.

No trace of the enzyme was found in Jensen sarcoma, nor was there any significant difference between normal livers and those from rats bearing the sarcoma.

As the data show, neither mouse liver nor mouse hepatomas exhibited any detectable *d*-amino acid oxidase activity. That the enzyme occurs in the species is shown by its presence in mouse kidney.

Certain crucial points were checked by measurements of ammonia production. The results agree with those for oxygen consumption. However, the deviation of the NH_3/O_2 ratio of 1.47 found for normal liver from the theoretical value of 2 (expected from Kreb's work on the isolated enzyme) should be noted. Since pyruvate is not oxidized under these conditions, it may indicate a binding of NH_3. Since the main conclusions are in no way affected by this discrepancy, the point was not investigated further.

DISCUSSION

What relation do the facts presented previously in this paper bear to the metabolism of tumor tissue, in particular to utilization of oxygen?

Respiration through *d*-amino acid oxidase is quantitatively not very important for either liver or hepatoma, representing only about one-thirtieth of the total respiration of liver. As to its qualitative significance, very little can be said, since its substrates, the *d*-amino acids, do not normally occur in the diet. Its absence in mouse liver and hepatomas in spite of their higher riboflavin content as compared with rat tissues is puzzling. Unless

one assumes that the presence of the enzyme in hepatic tissues is without biologic significance, these facts may indicate a real difference in the biochemistry of rat and mouse tissues. They serve to emphasize the dangers of combining isolated data obtained on many species into a comprehensive picture.

When rat liver becomes neoplastic, the *d*-amino acid oxidase activity is markedly reduced. This reduction, like that of xanthine oxidase (*1*), parallels the lowering of total riboflavin (*2*). However, the low *d*-amino acid oxidase activity of liver tumors is not necessarily a consequence of lower total riboflavin since less than 1γ/gram of riboflavin-adenine-dinucleotide[3] could account for the observed *d*-amino acid oxidase activity of liver if it were all in the form of amino acid oxidase. Furthermore, the lowering of *d*-amino acid oxidase activity might be caused by a deficiency of the protein component. Klein (*21*) showed that the increase in *d*-amino acid oxidase of liver caused by thyroid feeding was attributable to an increase in the specific protein rather than in riboflavin. A similar study with hepatoma is planned to determine whether the protein moiety is also lowered as compared with liver.

The cytochrome oxidase values ($Q_{O_2}^{ox}$) for the tumors studied fell between 2.61 and 7.4. These values are close to those previously reported for several tumors by Stotz (*8*) and Elliott and Greig (*23*). Although certain normal tissues, such as large intestine and diaphragm muscle (*8*) possess lower cytochrome oxidase activities than do any of the tumors, it does appear that tumors are low in this oxidase when the value of 30 for liver and 70 for heart are considered.

[3] *Based on the activity of the purified enzyme reported by Warburg and Christian* (20).

The question arises whether tumors are deficient in cytochrome oxidase or in the complete cytochrome system. A definite answer would require knowledge of energy requirements of the tissue for both maintenance and growth, knowledge which unfortunately is not available. However, it is unlikely that tissues possess any appreciable excess of respiratory enzymes in view of the onset of deficiency symptoms when the content of such components as thiamin or riboflavin is only slightly lowered.

It is apparent that the maximum cytochrome oxidase activities (with excess cytochrome *c*) are considerably greater than necessary to account for the respiration of tissue slices, for instance, 103 for liver and 38 for hepatoma 31 (on a dry-weight basis) as compared with Q_{O_2} values of about 6 (*24*). But in tissues cytochrome *c* is not present in excess, and consequently the maximum oxidase activity is not elicited. Assuming that oxidase and cytochrome interact as if in solution (i. e., neglecting the role of cellular structure), we can calculate maximum O_2 uptake via cytochrome oxidase-cytochrome *c* by using the Michaelis constant for the reaction given by Stotz (*25*). Stotz reported a value of 80γ cytochrome *c* per gram for liver, DuBois and Potter (*26*) of 90 for liver and 10 to 20 for a number of tumors not including transplanted hepatomas. By using Stotz's method, the values were found to be 80γ/gram for liver and 30 for hepatoma 31.[4] These figures lead to maximal O_2 uptakes through the oxidase-cytochrome *c* system of 42 for liver and 6 for hepatoma 31. Thus, it appears that oxygen consumption of tumor slices is really limited by the cytochrome system, whereas liver (and various other normal tissues) possesses

[4] *Unpublished data.*

considerable reserve. These results agree with the conclusions of Craig et al. (*27, p. 871*):

> When the homologous tissues were supplied with an adequate amount of succinate [or *p*-phenylenediamine] in addition to glucose, it became obvious that the normal tissues responded by increased oxygen consumption much more than did the malignant tissues.

Burk (*24*) pointed out that glycolysis is not a necessary accompaniment of growth inasmuch as the glycolysis of regenerating liver is no greater than that of normal liver. Nor is the Q_{O_2} value of regenerating liver, measured by the usual slice technique with glucose as substrate, greater than that of normal liver. However, the growth of tissue, as well as the respiration of a tissue slice, is limited not only by enzyme systems but also by available substrates. *In vivo* a wide variety of substrates and hormones needed by the growing tissue are constantly supplied. Liver, regenerating liver, and various other normal tissues could, in view of their cytochrome reserve, satisfy any additional energy requirements by increased oxidation. Since tumors do not possess an oxidative reserve, demands for more energy than used under tissue-slice conditions could be satisfied only by an increase in glycolysis.

SUMMARY

The activity of cytochrome oxidase and *d*-amino acid oxidase in a number of tumor and normal tissues was measured. Particular attention was given to tissues of hepatic origin.

The ratio of cytochrome oxidase activity of normal liver to hepatoma was about 4–5, to regenerating liver 1, to fetal liver 2.5, and to liver of tumor-bearing animals about 1.1. A variety of other tumors possessed low cytochrome oxidase activities of the same order of magnitude as hepatoma.

The ratio of *d*-amino acid oxidase activity of rat liver to hepatoma 31 was about 10, to regenerating liver 1.2, to fetal liver 7, and to liver of rats bearing hepatoma 31 about 1.5. Neither mouse liver nor mouse hepatomas exhibited measurable *d*-amino acid oxidase activity.

The significance of these results to oxygen utilization by tumor tissue is discussed.

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Further Studies of the Liver Catalase Activity of Tumor-Bearing Animals

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INTRODUCTION

The liver catalase activity of rats and of mice bearing rapidly growing tumors is considerably less than that of the livers of normal animals (*1–4*).[1] The effect of the tumor is reversible, for complete removal of the tumor either by surgical excision (*1, 2*) or by spontaneous regression (*2*) results in complete restoration of the liver catalase activity to the normal value. Partial removal of the tumor is followed by a temporary rise in the liver catalase activity which is roughly proportional to the amount of tumor removed (*1*). It is not known at the present time whether the effect of the presence of the tumor results in (1) a direct inhibitory effect on the catalase molecules present in the liver or (2) an inhibitory effect upon the mechanism which synthesizes the enzyme in this tissue. Both conditions could be brought about either by the liberation from the tumor into the blood stream of a toxic substance or substances, or by the abstracting by the tumor from the blood stream of components necessary for the normal maintenance or functioning of the liver catalase. The search for a solution of this problem is obviously difficult. According to alternative (1) the content of catalase is the same in the livers of normal and of tumor-bearing animals, but the presence of an inhibitor causes the activity in the latter animals to be much less than in the former. According to alternative (2) there is simply less total catalase in the livers of tumor-bearing animals. The most direct approach to the study of alternative (2) would be to isolate quantitatively the catalase from the livers of normal and of tumor-bearing animals. This is impracticable in the case of laboratory animals.

Only alternative (1) offers a few approaches, which, involving the search for the presence of an inhibitor, are necessarily indirect. Of these, the following have been employed: the incubation of fresh extracts of tumor with extracts of liver, the incubation of tumor-tissue slices with slices of liver, the incubation of serum and various tissues of tumor-bearing animals with normal liver, the dialysis of extracts of the livers of normal and tumor-bearing animals, and the kinetic study of diluted normal liver-extract preparations. In addition, some studies are reported of the effect of intraperitoneal injections of fresh tumor extracts on the liver catalase activity of normal rats. The incubation studies are designed to see whether there is any direct action of soluble tumor tissue components on liver catalase. The dialysis studies should show whether the inhibitory agent, if present, is dissociable. The kinetic studies are designed to see whether the rate of enzymatic reaction of diluted normal liver extracts is identical with that of liver extracts of tumor-bearing animals; if so, it would strongly suggest that there is simply less total catalase present in the latter tissue, for the presence of an inhibitor should be readily revealed by such studies. Indeed, earlier work on this subject (*3*)

[1] *Other liver enzymes such as arginase, xanthine dehydrogenase, amylase, and acid and alkaline phosphatase have practically the same activity in normal and in tumor-bearing mice.*

showed that the rates of catalase reaction at different dilutions of extracts of normal and of tumor-bearing animals were essentially similar, for the ratios of k, the calculated monomolecular reaction constant, at the same dilution for both extracts were practically identical. However, no attempt was made at that time to study extracts of the two kinds of tissue under condition of equal catalase activity. Study of such isocatalase systems are reported in this paper.

EXPERIMENTAL PROCEDURE

The tissues employed in these studies were obtained from normal Buffalo rats, normal dilute brown mice, Buffalo rats bearing the transplanted Jensen sarcoma, and dilute brown mice bearing the transplanted sarcoma 37. The tumor-bearing animals were killed by decapitation 2 weeks after the tumor had been inoculated, at which time the Jensen sarcoma weighed 20 gm. and sarcoma 37, 6 gm., on the average. Normal and tumor-bearing groups of animals were of the same sex (female) [2] and approximately of the same age. The tissues were used immediately after death of the animals. The details of preparing the tissues for catalase activity determinations and the technique used have been described (*1–4*). Whole tumors including the necrotic areas were used in the present studies.

Incubation of Tumor and Liver Extracts and Slices

The extracts represented 100 mg. of fresh tissue per cubic centimeter. For the incubation each cubic centimeter of the extract was diluted to 3 cc., and 1 cc. of the mixture, representing about 33 mg. of tissue, was used for the catalase determination. When two different tissues were employed in these incubation mixtures the amount of soluble tissue components was of course doubled. The extracts for the most part were prepared in distilled water, but some samples were made up in 1 percent sodium chloride and a few in rat serum. The tissue slices were cut with a razor moistened with the appropriate extract fluid and were weighed in 4 cc. of rat serum. The incubation of the extracts was carried out at 5° and at 36° C. in lightly stoppered tubes for 24 hours. The incubation of the tissue slices was carried out with continual shaking for 6 hours at 36° in the presence of a 95 percent oxygen-5 percent carbon dioxide gas mixture. After incubation, the slices were ground to a pulp in a glass mortar and taken up in the serum used to suspend the slices during the reaction. The catalase activity was determined on 1 cc. of this extract. In experiments in which the sarcoma slices were included in the incubation mixture, the tumor slices were ground together with the liver slices since the contribution of the former to the total catalase activity of the mixture was negligible. The extracts were all brought to 25° before the catalase activity was determined. For these particular studies, the enzymatic activity was estimated by the volumetric method (*3*). The results are given in table 1.

Table 1 shows that there is no direct effect of the tumor extracts or of the tissues of tumor-bearing animals on the catalase activity of normal liver.[3] No effect was observed on incubating tumor slices with normal liver slices. Although the extracts of the livers of the tumor-bearing animals had the usually low catalase activity, incu-

[2] *The effect of the presence of a tumor on the liver catalase is exactly the same for animals of both sexes.*

[3] *There is likewise no effect whatever of tumor extracts on the activity of crystalline beef catalase.*

TABLE 1.—*The catalase activity of mixtures of liver and tumor extracts and slices*

[Enzymatic activity was determined at 25° C. One cubic centimeter of the mixture was treated with 5 cc. of M/5 phosphate buffer at pH 6.9 and 1 cc. of 29 percent hydrogen peroxide]

Preparation [1]	Extract fluid	Incubation temperature	Evolution of oxygen
		°C.	*Cubic centimeters per second*
1 cc. rat L extract+1 cc. rat S+1 cc. water	Water	5	1.7
Do	do	36	1.6
1 cc. rat L extract+2 cc. rat S	do	5	1.8
Do	do	36	1.8
2 cc. rat S+1 cc. water	do	5	0
Do	do	36	0
2 cc. rat TS+1 cc. water	do	5	0
Do	do	36	0
1 cc. rat L extract+1 cc. rat TS+1 cc. water	do	5	1.5
Do	do	36	1.7
1 cc. rat L extract+2 cc. rat TS	do	5	1.7
Do	do	36	1.7
Do	TS	5	1.6
Do	TS	36	1.5
1 cc. rat L extract+2 cc. water	Water	5	1.8
Do	do	36	1.9
Do	S	5	1.7
Do	S	36	1.7
1 cc. rat L extract+2 cc. Jensen sarcoma extract	Water	5	1.8
Do	do	36	1.8
Do	S	5	1.4
Do	S	36	1.5
Do	TS	5	1.7
Do	TS	36	1.7
Do	Saline	5	1.4
Do	do	36	1.4
2 cc. Jensen sarcoma extract+1 cc. water	Water	5	0
Do	do	36	0
Do	S	5	0
Do	S	36	0
1 cc. rat L extract+2 cc. normal rat spleen extract	Water	5	1.8
Do	do	36	1.8
1 cc. rat L extract+2 cc. extract of spleen of tumor-bearing rats	do	5	1.7
Do	do	36	1.6
2 cc. normal spleen extract+1 cc. water	do	5	.004
Do	do	36	.002
2 cc. extract of spleens of tumor-bearing rats+1 cc. water	do	5	.002
Do	do	36	.002
0.5 cc. rat L extract+2.5 cc. water	do	5	.6
Do	do	36	.6
0.5 cc. rat TL extract+2.5 cc. water	do	5	.08
Do	do	36	.08
0.5 cc. rat L extract+0.5 cc. rat TL extract+2 cc. water	do	5	.8
Do	do	36	.9
1 cc. rat TL extract+2 cc. water	do	5	.2
Do	do	36	.2
1 cc. rat TL extract+2 cc. Jensen sarcoma extract	do	5	.2
Do	do	36	.2
Do	TS	5	.2
Do	TS	36	.2
1 cc. mouse L extract+2 cc. sarcoma 37 extract	Water	5	2.0
Do	do	36	2.1
2 cc. sarcoma 37 extract+1 cc. water	do	5	0
Do	do	36	0
1 cc. mouse TL extract+2 cc. water	do	5	.15
Do	do	36	.18
1 cc. mouse TL extract+2 cc. sarcoma 37 extract	do	5	.17
Do	do	36	.17
150 mg. rat L slices+4 cc. rat S		36	2.0
145 mg. rat L slices+110 mg. Jensen sarcoma slices+4 cc. rat S		36	2.1
105 mg. Jensen sarcoma slices+4 cc. rat S		36	.008

[1] L, livers of normal animals; TL, livers of tumor-bearing animals; S, serum of normal animals; TS, serum of tumor-bearing animals.

bation with extracts of the sarcoma resulted in no further change of the enzyme activity. The effect of adding equal amounts of extracts of the livers of normal and tumor-bearing rats was merely additive. A few of the experiments were made at 48 hours' incubation with no essential change in the results obtained at 24 hours.

Injection of Tumor Extracts

Several samples of transplanted hepatoma 31 (including necrotic areas) were ground up and extracted with one and one-half times the amount of 0.9 percent saline solution. After they had been stirred gently for 5 minutes, the extracts were centrifuged, and the residue was discarded. Two- and one-cc. aliquots of the supernatant were injected intraperitoneally into normal rats weighing about 100 gm. each. Simultaneously, a group of rats was injected similarly with either saline or with the serum of tumor-bearing rats. At intervals of ½ hour, 1, 2, 3, 6, 24, and 48 hours following the injection the rats were decapitated, and the liver catalase activity was determined. At no stage was there any difference in the liver catalase activity of the test and control groups of animals.[4]

Dialysis Experiments

Extracts of the livers of normal and sarcoma-bearing rats were made up in water and in M/5 phosphate buffer (pH 6.9) and dialyzed at 5° C. in cellophane bags against water and M/5 phosphate buffer, respectively. The catalase activity of the extracts was measured at 24- and 48-hour intervals and compared with that of the undialyzed extracts maintained under the same conditions. The extracts represented about 33 mg. of fresh wet tissue per cubic centimeter. The results are given in table 2.

There was little, if any, change in activity of the control extracts on standing up to 48 hours at 5° C. On the other hand, the activity of the dialyzed extracts dropped to about a third after 24 hours and to about an eighth after 48 hours. There was no significant difference between the extracts dialyzed in water and in the buffer. The important fact is that the proportionate drop in activity on dialysis was the same for the liver extracts of the normal and of the tumor-bearing animals (last column, table 2).

The outside fluid of the extracts dialyzed against water was evaporated at 40° C. *in vacuo* to about 5 cc. Addition of this concentrate to the dialyzed extract made no more change in the catalase activity of the mixture than was produced by the addition of an equal volume of distilled water.

The drop in catalase activity during dialysis is apparently caused by a progressive denaturation of liver proteins by this procedure. It is not a question of standing, since the activity of the undialyzed controls was practically constant up to at least 48 hours.

Kinetic Studies

Aqueous extracts of 33 mg. of fresh liver tissue per cubic centimeter were prepared from the livers of normal and of tumor-bearing rats. By the volumetric method of catalase determinations (*3*) the rate of oxygen evolution for the liver extract from normal animals was 1.6 cc. per second, for the liver extract from tumor-bearing animals the rate was 0.2 cc. per second. Half of the volume of the extract of the liver of normal rats was placed in a boiling water bath for 5 minutes. At the end of this time the extract was cooled to room temperature. Only a trace of coagulum was present. Catalase activity was completely absent from this heated preparation. Small increments of the heated solution were added to the original extract of

[4] *A saline solution of crystalline beef catalase (prepared by Dr. J. Shack) with an enzymatic activity equal to that of one mouse liver was injected intraperitoneally every day into each of a group of mice, starting from the day the mice were inoculated with the transplanted sarcoma 37 until death of the animals about 2 weeks later. The catalase activity of the livers of these mice and their average survival rate were no different from those of untreated tumor-bearing mice.*

TABLE 2.—*Catalase activity of dialyzed extracts of the livers of normal rats and of rats bearing the Jensen sarcoma*

[Enzymatic activity was determined at 25° C. One cubic centimeter of the extract was treated with 5 cc. of M/5 phosphate buffer at pH 6.9 and 1 cc. of 29 percent hydrogen peroxide]

Extract [1]	Extract and dialysis fluid	Dialysis period (test)	Standing period (control)	Evolution of oxygen	Ratio of oxygen evolution of test to control
		Hours	*Hours*	*Cc. per second*	
L	Water	0	0	1.8	
L	Buffer	0	0	1.7	
TL	Water	0	0	.3	
TL	Buffer	0	0	.4	
L	Water	24		.5	
L (control)	do		24	1.7	3.4
L	do	48		.2	
L (control)	do		48	1.5	7.5
TL	do	24		.1	
TL (control)	do		24	.3	3.0
TL	do	48		.04	
TL (control)	do		48	.3	7.5
L	Buffer	24		.6	
L (control)	do		24	1.7	2.9
L	do	48		.2	
L (control)	do		48	1.7	8.5
TL	do	24		.1	
TL (control)	do		24	.3	3.0
TL	do	48		.05	
TL (control	do		48	.3	6.0

[1] L, livers of normal rats; TL, livers of tumor-bearing animals.

normal liver until the catalase activity of the mixture was equal to that of the extract of the livers of tumor-bearing rats. When exactly 1.2 cc. of the heated extract was added to 1 cc. of the original extract, 1 cc. of this mixture produced an oxygen evolution rate equal to that of 1 cc. of the liver extract of tumor-bearing rats, or 0.2 cc. per second. In other words, dilution of the normal liver extract by little more than 100 percent reduced its activity by several hundred percent. This reduction in activity has been observed previously (*1–4*).

Two extracts of equal catalase activity were thus prepared, one made up of diluted normal rat liver extract and the other of undiluted extract of livers of tumor-bearing rats. For kinetic studies, the volumetric method of catalase activity determinations is too insensitive, and it was necessary to use a titration method. The latter method has already been described (*3*). The extracts of equal catalase activity were further diluted with water for these studies as follows: 1:3, 1:6, 1:12, and 1:30. The initial substrate concentration was the same for each dilution. The results were calculated according to the monomolecular reaction formula, and calculated values of k, the reaction constant, are given in table 3.

Table 3 shows that the rates of reaction for the extracts of equal catalase activity of normal liver and of the livers of tumor-bearing animals are very nearly identical over a tenfold range of dilution of the extracts. The rate for these extracts does not appear to follow a monomolecular course except at high dilution. At equal times of incubation, the monomolecular reaction constant, k, falls off sharply with dilution. Thus at a dilution of 1:3, $k \times 10^4$ is 777 for 10 minutes' incubation, while at 1:6 it is 384, at 1:12 it is 130 (average), and at 1:30 it is 30.

The original normal liver extract when diluted 1:2.2 dropped eightfold in catalase activity. The latter was estimated by the rate of oxygen gas evolution at 25° C. Further dilution of this extract resulted in a decrease in activity which was meas-

TABLE 3.—*Kinetics of liver extracts of equal catalase activity of normal and of tumor-bearing rats*

[50 cc. of 0.01 N hydrogen peroxide in 0.015M phosphate buffer incubated at 0° C. with 1.0 cc. liver extract; aliquots of 5.0 cc removed at intervals and treated successively with 10 percent sulfuric acid, 10 percent potassium iodide, and 1 percent ammonium molybdate; finally the liberated iodine was titrated with 0.05N thiosulfate]

Dilution (with water)	Time of incubation	Normal liver extract (33 mg. tissue per cubic centimeter) diluted 1-2.2 with boiled normal liver extract		Liver extract (33 mg. tissue per cubic centimeter) from tumor-bearing rats	
		Splitting of H_2O_2	k	Splitting of H_2O_2	k
	Minutes	*Percent*	$x10^4$	*Percent*	$x10^4$
1-3	5	36	887	35	860
	10	54	777	54	777
	15	64	664	62	640
1-6	5	21	478	22	492
	10	32	384	32	384
	15	42	357	40	330
	20	47	328	47	328
	25	51	278	53	295
	30	55	242	57	256
1-12	5	8	188	8	188
	10	12	121	13	138
	15	15	102	15	102
	20	16	91	17	99
	30	21	73	20	70
	40	24	70	24	70
	60	30	62	30	62
1-30	10	3	30	3	30
	20	5	25	5	25
	30	7	20	7	20
	40	8	20	8	20
	50	9	21	9	21
	60	10	19	10	19
	90	12	18	12	18
	120	18	18	17	17

ured under different conditions at 0° and expressed in terms of the logarithmic monomolecular reaction formula. The measured drop in activity by the two different methods, i. e., volumetric and titration, is thus not comparable. This point has been discussed earlier (*3*).

The considerable drop in liver catalase activity on dilution or on dialysis indicates the lability of this enzyme in the presence of other tissue components. Crystalline beef catalase is relatively stable, and in reaction it closely approximates the monomolecular course.[5]

Extracts of normal rat liver and of the livers of tumor-bearing animals were prepared in M/5 phosphate buffer, and the liver extract of the former was brought to the activity of the latter, in the manner previously described, with boiled liver extract. The extracts of equal catalase activity were then dialyzed for 48 hours at 5° C. against M/5 phosphate buffer. The drop in activity for both extracts was the same at the end of this period and of the same magnitude as that given in table 2.

[5] *Unpublished observations.*

DISCUSSION

The failure to find any effect whatever of preparations of tumors and of the tissues of tumor-bearing animals on the catalase activity of the liver is strong presumption that the decrease in the liver catalase activity of tumor-bearing animals is not a result of a direct inhibition by tumor products, of the liver catalase. The fact that the proportionate drop in activity on dialysis is the same for extracts of normal livers and of the livers of tumor-bearing animals indicates likewise the absence of a dissociable inhibitor, and hence of directly reversible inhibition. The iden-

tical kinetic behavior of the enzyme in both kinds of liver also favors this view. Admittedly, all this evidence is of a negative character. Nevertheless, it permits, for the time being at least, the elimination of the idea of direct inhibition of the liver catalase as a major explanation.

The alternative explanation is that the mechanism for the synthesis of catalase is somehow interfered with in the livers of tumor-bearing animals. The result is that there is less total catalase present in the livers of tumor-bearing animals. The identical kinetic behavior of the liver extracts (table 3) might be interpreted on the basis that the livers of tumor-bearing animals have a little less than half the total catalase content of normal livers.

It is highly suggestive that, so far as we know at the present time, two of the most marked systemic effects that occur in nearly all tumor-bearing animals are (1) a decrease in liver catalase activity, and (2) a decrease in hemoglobin (*5*). Both catalase and hemoglobin are conjugated proteins containing the same hematoporphyrin prosthetic group. Only the protein in each molecule is different. It is possible, therefore, that the systemic effect produced in tumor-bearing animals is concerned with an interference with the synthesis of the hematoporphyrin nucleus.

The injection of tumor extracts into normal animals does not result in any observable change in the liver catalase activity of these animals. It seems probable that the living tumor in the animal is a necessary condition to the effect which is produced on the liver catalase mechanism.

SUMMARY

Extracts of normal rat liver were incubated at two different temperatures with extracts of transplanted Jensen sarcoma, and with serum and extracts of other tissues of tumor-bearing rats. Similarly, rat liver slices were incubated with tumor slices, and extracts of normal mouse liver with extracts of sarcoma 37. No effect on the catalase activity of the livers was observed in any of these mixtures. There is apparently no direct action *in vitro* of tumor tissue on liver catalase.

Extracts of normal rat liver and of the livers of tumor-bearing rats were dialyzed at low temperatures for 24 and 48 hours. The catalase activity of both extracts dropped during the dialysis, but the proportionate drop of each extract was the same throughout. This fact appears to rule out any readily dissociable inhibitor combined with the catalase in the livers of the tumor-bearing animals.

Aqueous extracts were prepared at the same concentrations from livers of normal and of tumor-bearing rats. When the extracts from the livers of normal animals were diluted with boiled normal liver extract in the ratio of 1:2.2, the catalase activity measured by oxygen evolution of the diluted normal liver extracts and of the undiluted liver extracts of the tumor-bearing animals was the same. The rates of reaction with hydrogen peroxide of such extracts were practically identical, and the drop in activity in each on dialysis was the same.

Extracts of a transplanted hepatoma injected intraperitoneally into normal rats produced no change in the liver catalase activity of these animals.

In the light of these findings the view is advanced that the low liver catalase activity of tumor-bearing animals is ascribable to the presence of only about half the normal amount of total catalase in this organ. Since the hemoglobin concentration in tumor-bearing animals is also markedly lowered, it is suggested that this interference with catalase and hemoglobin synthesis is concerned with the formation of the hematoporphyrin nucleus.

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Note on the Copper Content of the Tissues of Tumor-Bearing Animals

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Copper is an essential element in the metabolism of animals. Its relation to the problem of cancer has been little considered. In order to see whether the distribution of this element is altered in tumor-bearing animals, we have adopted as a first approach a comparison of the copper content of certain tissues of these and of normal animals.

EXPERIMENTAL PROCEDURE

Tissues

For this study the following tissues were used: livers and kidneys of normal Buffalo rats, of normal Osborne-Mendel rats, and of normal dilute brown and of C3H mice; the same tissues of rats bearing the transplanted Jensen sarcoma and the transplanted hepatoma 31 (*1*), and of mice bearing transplanted sarcoma 37, transplanted hepatomas 1 (*2*) and 98/15 (*3*), and spontaneous mammary tumors. The average weights of the tumors and the time elapsed since their implantation are as follows: Jensen sarcoma, 35 gm. and 2 weeks; hepatoma 31, 40 gm. and 6 weeks; sarcoma 37, 5 gm. and 2 weeks; hepatoma 1, 3 gm. and 3 weeks; hepatoma 98/15, 2 gm. and 2 months. The spontaneous mammary tumors weighed on the average 7 gm. apiece and were studied about 8 weeks after they were first palpable in the animal. Animals bearing the Jensen sarcoma and sarcoma 37 do not survive for many days after the period given. It is necessary to give the tumor weights and the time elapsed since implantation because it has been found that the systemic effects elicited in tumor-bearing animals often depend upon the rate of growth of the tumor (*4*, *5*). Slow-growing or regressing tumors cannot be used for studies of systemic effects. In addition, the necrosis-free and necrotic portions (determined grossly) of rat hepatoma 31 transplants were investigated as well as transplants of mouse hepatomas 1 and 98/15. Studies of the serum and citrated blood of normal rats and of rats bearing the Jensen sarcoma were also included. All animals were fed on a Purina dog-chow diet.

The animals were killed by decapitation. The tissues were immediately removed and were ground in a glass mortar, and an amount was weighed in a flask provided with a ground-glass stopper. Twice the weight of acetone was added, and the material stirred occasionally for an hour. The acetone was driven off in a water bath and the flask with its contents heated at 105° C. in an oven for 24 hours. At the end of this time, the flask was weighed. The heating was then continued until constant weight was achieved. The maximum difference in weight gave the amount of water in the tissue. Dry ether was added to the flask, boiled on a hot plate, and decanted. This process was repeated five times with ether and six times with dry petroleum ether. The flask was then heated in the oven until the weight was contant. The loss in weight gave the amount of lipoid present in the tissue. The amount varied considerably. The residue which was water- and lipoid-free was used for the copper determinations.

Copper Determinations

An amount of the dried, defatted residue, generally 0.2 to 0.5 gm., was placed in a thick-walled test tube, and 1 cc. of concentrated sulfuric acid and 3 cc. of 60 percent perchloric acid were added. The contents of the tube were then boiled over a free flame until only sulfuric acid fumes appeared. Ten cubic centimeters of distilled water was added plus 5 cc. of 4 percent sodium pyrophosphate solution in water. A small piece of red litmus paper was added and then ammonia until the mixture was just alkaline.[1] The tubes were cooled and treated with 10 cc. of amyl alcohol and 0.5 cc. of a 2-percent solution of sodium diethyldithiocarbamate. The contents of the tube were transferred to a separatory funnel which was strongly shaken. The amyl alcohol layer was separated, filtered through dry filter paper, and compared in a colorimeter against a standard solution of copper sulfate (0.001 mg. Cu per cubic centimeter in water) treated in exactly the same fashion. A blank run of the reagents was made. Determinations of the copper in blood and in serum were made by the ashing procedure of Tompsett *(6)*.

Determinations of copper on the same sample of tissue agreed within 2 percent. Determinations of nine individual rat livers in the Buffalo and of nine in the Osborne-Mendel groups gave a variation of no more than ± 10 percent between one animal and the other. The kidneys of the nine rats in each group were divided into three pools, and each pool was analyzed separately. The copper content of the pools varied by no more than ± 10 percent. Eight mice in each group of normal and of tumor-bearing animals were studied. The livers and kidneys of the mice of each group were segregated into pools of four livers or pairs of kidneys each. Thus the tissues of each group of mice were run in duplicate. The copper value of the duplicates in each group differed by 5–10 percent. Similar determinations of the copper content of the hepatomas yielded values which differed by 4–7 percent. The blood and serum were obtained from pools of four animals each out of eight animals in each group of normal and of tumor-bearing rats. The duplicates agreed within 12 percent. The results on the blood and serum are given in the following tabulation:

Tissue	*Copper content in milligrams per 100 cc.*
Serum of normal Buffalo rats	0.32
Serum of Buffalo rats bearing transplanted Jensen sarcoma	.26
Citrated blood of normal Buffalo rats	.43
Citrated blood of Buffalo rats bearing transplanted Jensen sarcoma	.28

The copper content of the whole blood of normal animals is only slightly higher than that of serum which implies that the copper is not far from being equally distributed between cells and serum. Whereas the copper content of the serum of tumor-bearing rats is only slightly lower than that of normal rats, the copper content in the whole blood of the former group of animals is distinctly lower than that of the latter group. The results of the studies on the various tissues are given in table 1.

The copper content of normal and regenerating rat livers is the same, but that of the livers of tumor-bearing rats is distinctly elevated over the normal. The copper content of the normal mouse livers is considerably higher than that of normal

[1] *Under these conditions iron does not interfere with the copper determinations. This was tested by running controls containing, in addition to copper, a wide range of ferric chloride concentrations.*

TABLE 1.—*Copper content of transplanted hepatomas and of the tissues of normal and of tumor-bearing animals*

Tissue	Sex	Copper content—	
		Per 100 mg. dry lipoid-free tissue	Per 100 gm. wet tissue
		Milligrams	*Milligrams*
Livers of normal Osborne-Mendel rats	F	2.5	0.68
Do	M	2.5	.70
Regenerating livers of Osborne-Mendel rats	M	2.5	.66
Livers of Osborne-Mendel rats bearing transplanted hepatoma 31	M	4.0	.92
Transplanted hepatoma 31 in Osborne-Mendel rats	M	2.0	.38
Necrotic area of transplanted hepatoma 31 in Osborne-Mendel rats	M	2.9	.61
Livers of normal Buffalo rats	M	2.5	.70
Do	F	2.5	.68
Livers of Buffalo rats bearing transplanted Jensen sarcoma	F	4.0	.94
Kidneys of normal Buffalo rats	F	7.0	1.51
Kidneys of Buffalo rats bearing transplanted Jensen sarcoma	F	4.0	.83
Livers of normal dilute brown mice	F	3.1	.91
Livers of dilute brown mice bearing transplanted sarcoma 37	F	4.0	.99
Livers of dilute brown × I mice bearing transplanted hepatoma 1	M	3.6	.91
Transplanted hepatoma 1 in dilute brown × I mice	M	1.8	.33
Livers of normal C3H mice	F	3.1	.85
Livers of C3H mice bearing transplanted hepatoma 98/15	F	5.0	1.11
Transplanted hepatoma 98/15 in C3H mice	F	2.4	.47
Livers of C3H mice bearing spontaneous mammary tumors	F	3.5	.88
Kidneys of normal C3H mice	F	5.0	1.15
Kidneys of C3H mice bearing spontaneous mammary tumors	F	4.2	.97

rat livers, but the increase in copper in the livers of tumor-bearing mice over that of the normal is not so great as in rats. Indeed, the difference between the copper content of the livers of normal and of tumor-bearing mice is discernible only on the basis of the dry weight of tissue, for on the basis of wet weight these differences tend to disappear. The reason for this is probably owing to the higher water content (7) of the livers of the tumor-bearing mice and the relatively small increase in copper.

The kidneys of normal rats and mice have a higher copper content than the livers of these species. In tumor-bearing animals, however, the copper content of the kidneys decreases, in contrast with the liver in which the copper content increases as compared with that of normal animals.

The greater drop in the copper content of whole blood as compared with that of serum in rats bearing the Jensen sarcoma can be interpreted for the most part as being attributable to the anemia which generally accompanies tumor growth. It has been shown (5) that the red cell count of the sarcoma-bearing rats is about 30 percent lower and that of the hemoglobin concentration about 20 percent lower than the respective values found in the blood of normal rats. Taylor and Pollack (8) have reported that the hemoglobin level of tumor-bearing mice falls sharply during growth of the tumors. With fewer red cells in the blood of the tumor-bearing animals, the copper content of the whole blood might be expected to be lower than that of normal animals. The value reported herein for the copper of normal rat serum falls within the range of values reported by Warburg and Krebs (9) who used a different method of analysis.

The distinct rise in the copper content of livers of tumor-bearing animals is more difficult to interpret. The total solids of such livers is lower than those of normal animals. It is possible that the liver functions as a distributing center for copper, and when for some reason it cannot send this element into the blood and thence to other body organs such as the kidneys,

spleen, etc., the copper accumulates in the liver.[2]

In a study of the copper content of the livers of 17 human beings who died as a result of different types of cancer, Gerlach (*10*) found that 6 gave elevated values for copper and 11 gave normal values. This investigator used the wet tissue in conjunction with a spectrographic method for copper and reported his results on a wet-tissue basis. It is possible that if he had performed his analyses on the dried, lipoid-free residue, a larger proportion of the livers would have been found to contain elevated values for copper since the higher water content would tend to mask any relatively small increase in copper (table 1). Edlbacher and Gerlach (*11*) found that of the livers of 3 rats bearing the Jensen sarcoma for 4 weeks, 2 showed considerably elevated copper values and 1 gave a normal value. Recently, Sandberg, Gross, and Holly (*14*) found that excessive storage of copper in the liver takes place in various forms of human cancer. The increase in the copper content of the liver appears to be common to cancerous animals and human beings.

[2] *We have evidence that the spleens of the tumor-bearing rats and mice have considerably less copper than those of normal animals. The data are not reported in table 1 because of some interference of the hydrolysates of this tissue with the color developed by the diethyldithiocarbamate reagent. Since accurate determinations of these tissues were not possible by the technique used, we mention here the results of semiquantitative observations.*

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Peculiar Growth Lesions in Frogs Induced by Irradiation of Sperm Cells With X-Rays

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INTRODUCTION

In 1930 Witschi (*1*) described certain unusual growths that arose in frogs during embryonic development. These growths were obtained by allowing eggs to become overripe before fertilization and were found to possess growth properties that in some respects simulate those of neoplasms. These findings have such interest in connection with tumor etiology that it is important to consider the subject further and if possible to supplement the information now available.

Normally, eggs remain in the ovary of the female frog during the winter months, and as the spring spawning season approaches, they break through the walls of the ovary, move freely in the abdomen, and eventually enter the open ends of the oviducts just beneath the diaphragm. They then pass along the tortuous ducts undergoing further maturation as they do so and come to rest in a dilated section near the cloaca, called the uterus. Ordinarily, the female frog is clasped by the male at this time, and the eggs are forced into the water where they become fertilized by sperm shed by the male.

Under normal conditions, transfer of eggs from ovary to uterus takes place in a few hours, and as a rule the eggs are shed and fertilized within the next 2 or 3 days. Ordinarily, normal embryos will result if fertilization occurs during this period, but as Witschi and others (Briggs (*2*), Briggs and Berrill (*3*), Zimmerman and Rugh (*4*)) have pointed out, grossly abnormal embryos may result if the eggs lie in the uterus more than 3 days.

Abnormalities reported by the various workers are of the most bizarre types: (1) Polyspermy may occur, with the resulting multiple segmentation of the egg at the time of the first cleavage; (2) there is a tendency toward axial duplications and accessory appendages with the consequent formation of grotesque forms; and (3), most important from the standpoint of neoplasia, the power of differentiation in the embryonic cells becomes reduced and some of the cells enter a phase of abnormal growth.

Histologic sections of abnormal embryos show a striking display of undifferentiated cells, and often only the smallest rudiments of organ parts such as brain lobes or optic cups can be found.

According to Witschi, mesenchyme and blood cells may assume unusual features and in some cases invade neighboring tissues. In addition to finding abnormal growths in the mesoderm, Witschi saw nodular masses in the endoderm and numerous papillomatous growths in the ectoderm. Some of the last-mentioned growths were observed to regress and disappear, whereas others were found to grow progressively until death of the tadpole occurred in a few days. Fragments of some of the nodules were transplanted into other tadpoles in some of which invasive growth was seen to take place.

Witschi described a young frog which had carried a strongly growing implant for 22 days. At necropsy a mass was found that infiltrated the viscera, and a nodule was seen above the heart which appeared to be metastatic. The heavily infiltrated tissues were transplanted, together with most of the viscera, into the body cavity of an adult frog. Sixty-two days later this animal was found to contain a large infiltrating and metastasizing tumor in the body cavity. Although interesting, this finding has the limitation of any isolated observation, and one cannot say how much the treatment contributed to the result.

Briggs and Berrill (*3*) investigated further the transplantability of frog papillomatous lesions induced by the overaging of eggs. These workers were able, as was Witschi, to obtain growths at the site of implantation into other tadpoles. Since under these circumstances it was impossible to distinguish transplant from host tissue, they decided to use the familiar procedure of transplanting pigmented donor tissue into nonpigmented host, and vice versa. By this means it was possible to determine whether the growths arose from the transplant or from host tissues. They were found to arise from host tissues and accordingly could not be designated as true transplants; instead it was necessary to consider them as induced growths.

Briggs and Berrill observed that such induced growths occurred only in organisms that did not grow and develop well. For this reason they concluded that the new growths were able to survive only when the vitality of the host was weak and that such growths were not true neoplasms.

Even though the growth characteristics of these lesions may not be in strict accord with the familiar criteria of malignant growth, this should not lessen interest in them or the factors that control their behavior, as information of this type has an important bearing on the understanding of growth control mechanisms generally. Moreover, as will become evident later, one cannot conclude with certainty that such lesions are not truly malignant.

It is important to point out that the same, or at least closely similar, abnormalities can be produced by various other treatments of frog eggs. Chemical and physical agents or conditions such as the following have been used successfully: 1,2,5,6-dibenzanthracene (*5*), 2,4-dinitrophenol (*6*), sucrose (*7*), anoxemia (*8*), low temperatures (*9,10*), sterols (*11*), ultraviolet and X-rays (*12, 13*). It should be emphasized, however, that in all the cases cited, the abnormalities were obtained by treatment of the egg and not the sperm.

Because of certain previous work with frog gametes, we were led to believe that abnormalities such as those described, including the odd growth lesions, might be obtained by irradiating sperm with X-rays. Thus, should this be accomplished, a number of interesting implications would at once be evident.

EXPERIMENTAL PROCEDURE

Frogs of the species *Rana pipiens* were employed. As the work was carried out during winter months, the animals were kept satisfactorily in running tap water until used (not more than 3 to 4 weeks at any time).

Eight to ten testes were minced in 5 ml. of fresh spring water. The sperm suspension thus obtained was divided into two or more parts, as the experiment required, each part being placed in a small celluloid box with cover. The samples were then ready for treatment.

Eggs were obtained by the now well-known pituitary technique. Pituitaries from two to four frogs were removed.

minced in 0.5 ml. of spring water, and injected into the peritoneal cavity of a female frog. An animal so treated and kept in running tap water at 18° to 20° C. had as a rule a supply of eggs in the uterus suitable for use in 24 to 48 hours. The eggs were made available simply by holding the animal firmly and squeezing the abdomen in such a manner as to cause the eggs to drop directly into the proper containers.

The X-ray treatment conditions were 186 kv. and 20 ma., the factors of filter and distance varying with the intensity needed.

Motility and Fertilizing Power of Irradiated Sperm Material

Tests were carried out to determine the amount of radiation required to stop completely the motility and fertilizing action of freshly collected sperm cells. Sperm material was placed under the X-ray machine for treatment. By employing two X-ray tubes in the same electrical circuit, arranging them so as to utilize both beams of radiation simultaneously, making the distance as short as possible (10 cm. from the center of each target), and using no filter, an intensity of radiation of approximately 7,000 r per minute was obtained. Doses of one, two, three, four, and five hundred thousand r were given, the time required for administering the largest dose being not more than 50 minutes.

Immediately after treatment, the sperm samples were examined microscopically for motility and tested for fertilizability. In order to accomplish the latter, the samples were each diluted with 5 ml. of spring water and placed in glass finger bowls, into which were squeezed 100 to 200 eggs. By having the finger bowls tilted slightly 5 to 6 ml. of sperm suspension was just sufficient to cover the egg masses. A small volume of sperm suspension was used in order to provide a greater concentration of the sperm cells available and thereby insure a greater likelihood of contact between sperm cell and egg. After about 15 minutes, the sperm suspension was poured off, and the eggs were covered with fresh spring water. In this condition, the material was watched for rotation of the eggs within the jelly membranes and for the first cleavages, both of which are criteria for fertilization. It was found that three to four hundred thousand r are required to stop motility of the sperm completely and at once.

This experiment was repeated 10 times, and essentially the same results obtained in every case, that is, almost no effect with 200,000 r, considerable inactivation with 300,000 r, and complete inactivation with 400,000 r. It was found, also, that any sample that contained motile sperm cells would cause fertilization in at least part of the eggs of a sample. From this, it was apparent that sperm cells capable of swimming were also capable of fertilizing eggs. It was also found that essentially the same conditions of motility and fertilizability existed 5 hours after irradiation. Beyond this time, however, the responses of both control and experimental material were variable.

Two points of interest arose in connection with the first cleavage: (1) When cleavage occurred in the experimental material, it occurred at the same time as in the controls. This is in contrast with results obtained with other material, such as sea urchin gametes (*14*), in which irradiation of sperm cells before fertilization causes a delay in the onset of the first cleavage. (2) The cleavage, when it occurred, was regular, that is, not multipolar in character, in contrast with the result obtained with overripe eggs (frog) in which multipolar cleavage is observed.

These results suggest that the polyspermic condition obtained by other investigators was solely an egg phenomenon, presumably associated with the egg cytoplasm since the egg contains such a vastly greater quantity of this component.

Developmental Abnormalities

When developmental changes were to be studied, sperm suspensions were prepared for irradiation as described previously. Different treatment conditions were used, however, since very much smaller doses were required. Only 1 X-ray beam was employed, and a filter of 0.25 mm. copper and 0.55 mm. aluminum was used. The distance was 25 cm., and the doses covered a range as follows: 0, 15, 25, 50, 100, 250, 500, 1,000, 10,000, and 50,000 r. After treatment the sperm samples were handled and allowed to fertilize normal eggs in the same manner as before. When the controls had developed to about the 10-mm. tadpole stage, the various samples appeared as shown in figure 1.

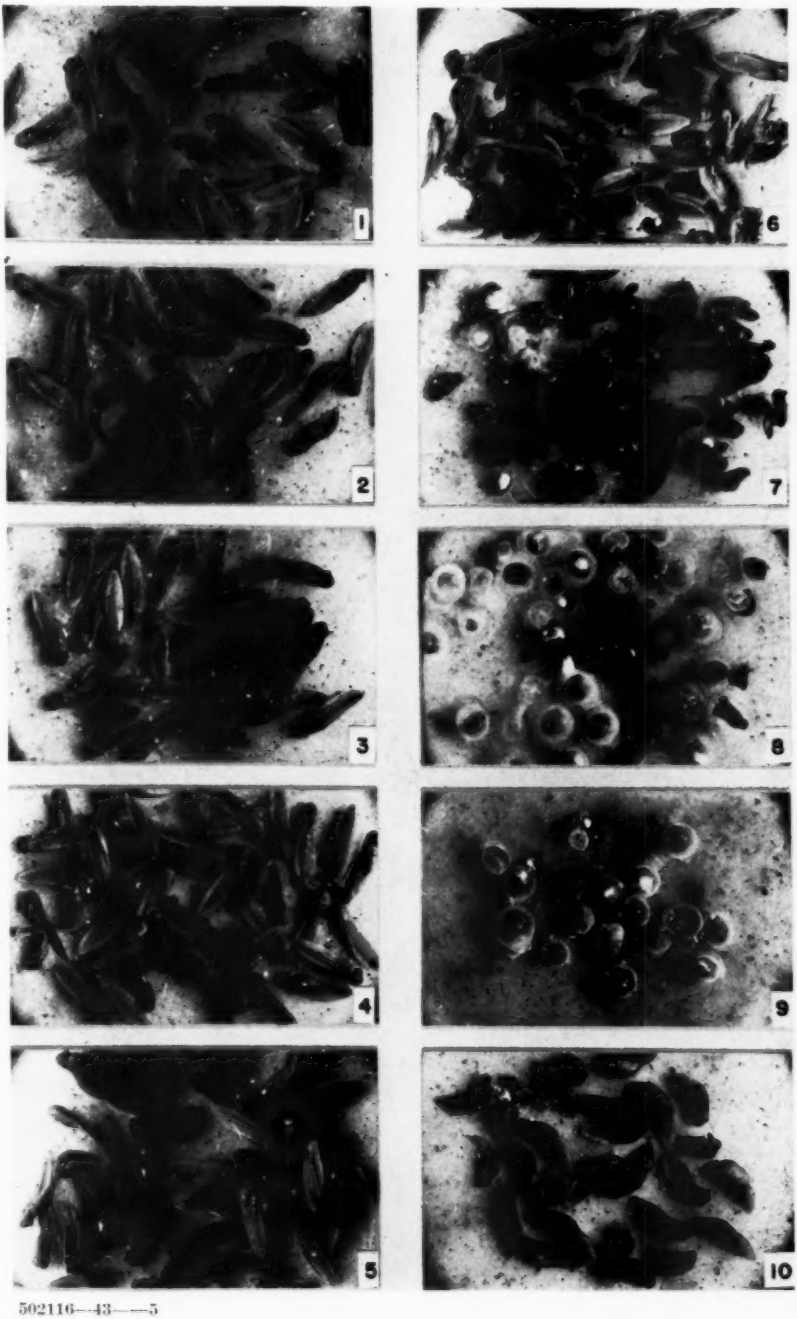
Extensive injury occurred in those samples the sperm of which received exposure to X-rays before fertilization, and for the most part the frequency of abnormality varied directly with the dose of radiation administered.

It is of interest that some abnormalities appear to be caused by comparatively very low doses (about 5 percent for 15 r) and that nearly all of the organisms show abnormalities when 500-r doses are used. Thus, whereas 300,000 to 400,000 r (to sperm) are required to stop motility and to prevent fertilization, 500 r or less (to sperm) will cause extensive developmental abnormalities in a large percentage of individuals (embryos and tadpoles)—a very striking difference in radiosensitivity as far as these two biologic processes are concerned. The implications arising from these findings are discussed later.

Figure 1 shows that the 50,000-r sample presents a peculiar variation in the trend of the results. The degree of injury per individual in the sample does not appear to be nearly so great as in the 10,000-r or even the 1,000-r samples. This observation, however, appears to be in accord with results presented previously (Hertwig (9) and others) which show that if the dose of radiation applied to sperm is large, yet not large enough to stop motility, the sperm will enter the egg in the usual way and stimulate it to development but will not take part in the subsequent nuclear activity of the cells. Development in such cases is gynogenetic, and the body shapes and contours of the 50,000-r forms (fig. 1) are typical of haploid organisms in this species. Thus, although no chromosome counts were made, it seems safe to conclude that haploid forms were being dealt with here.

With regard to abnormalities, figure 2 shows something of the character of the results obtained. Photographs 1 and 6 show, respectively, the intact animal and section of a control organism at the time of examination for injuries. At this stage, a marked degree of differentiation has taken place in the normal animal, and such organs as the eye, brain, gill arches, gut, etc., are plainly visible. Photographs 2, 3, and 4, on the other hand, show other organisms which were handled in exactly the same manner except that the sperm cells were exposed to a dose of 500 r of X-rays before fertilization. It is apparent at once that extensive modifications have occurred. Not only are there marked dwarfing and less distinct differentiation,

Figure 1.—Samples of frog embryos showing the nature and extent of abnormalities which develop following exposure of the sperm to X-rays. The doses of radiation applied were 0 (controls), 15, 25, 50, 100, 250, 500, 1,000, 10,000, and 50,000 r for samples 1 to 10, respectively.



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but also numerous peculiar ectodermal growth folds. Sections of such organisms (photographs 7 to 10) show that almost no differentiation has taken place in some cases, and that the head and tail polarity may be distinguished only with difficulty. Furthermore, it may be seen that the growth lesions are limited mainly or entirely to the ectoderm and are only a few cell layers thick at any point. These abnormalities, therefore, resemble closely, if indeed they are not identical with, those obtained by Witschi, by Briggs and Berrill, and others.

In connection with work on the treatment of sperm with X-rays, experiments were carried out on the overaging of eggs as had been done previously. Abnormalities were obtained which resembled in all important details those described by the other workers. Moreover, these abnormalities were indistinguishable in gross appearance and histologic detail from those obtained by treating sperm. This, of course, does not constitute final proof that the changes induced by treating sperm cells with X-ray are the same as those obtained by overaging eggs, but the findings are in accord with this view.

The specimen shown in photograph 5 (fig. 2) was obtained by the overaging of an egg. This is an example of the grossly peculiar forms encountered. The light peduncular projection of tissue at the lower left was set free by autoamputation shortly after this photograph was made. The amputated piece remained alive during the next 30 hours and enlarged somewhat. That it was alive during this time was easy to discern for the outer cells were ciliated and kept the mass in motion. This specimen showed abnormalities somewhat more extreme than any obtained by treating sperm, but it seems that little if any significance can be attached to this fact.

By way of summary, the following points may be listed:

(1) Extensive developmental abnormalities occurred in organisms the sperm of which received exposure to X-rays before fertilization.

(2) These abnormalities varied in type from slight organ deformity and duplication to almost complete lack of differentiation and abnormal growth. Among the latter were certain papillomatous growths of ectoderm which showed anaplasia but no invasiveness.

(3) The abnormalities appeared similar to, if not identical with, those obtained by applying various physical and chemical treatments to eggs.

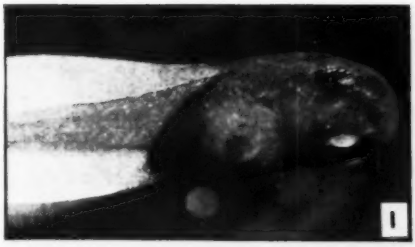
(4) The frequency of abnormalities varied directly with dose of radiation over a wide range.

(5) Whereas the abnormalities were produced by exposure of either gamete to doses of a hundred r or less, doses of three to four hundred thousand r were required to stop the motility and fertilizing ability of mature sperm.

DISCUSSION

These results have a bearing on three general problems: (1) Atypical cell growth; (2) biologic action of high energy radiation; and (3) germ-cell injury caused by high energy radiation.

FIGURE 2.—Photographs of individual whole embryos and of tissue sections. No. 1, an organism whose sperm had not been exposed to X-rays; No. 6, a tissue section of a similar organism; Nos. 2, 3, and 4, embryos approximately equal in age to the embryo in photograph 1, but whose sperm had been exposed to 500 r of X-rays before fertilization; No. 5, an organism which developed from an overaged egg and a normal sperm; Nos. 7 and 8, tissue sections of organisms generally similar to those in photographs 2, 3, and 4; Nos. 9 and 10, higher magnifications of the ectodermal overgrowths.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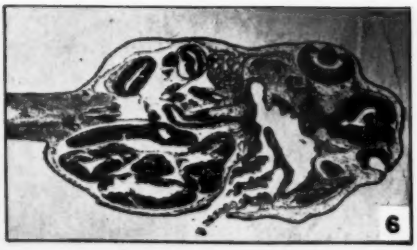
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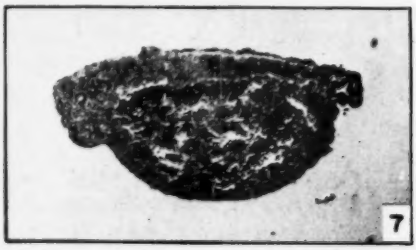
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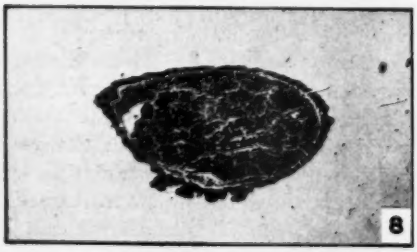
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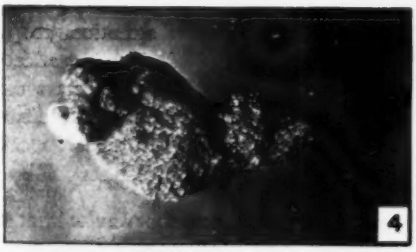
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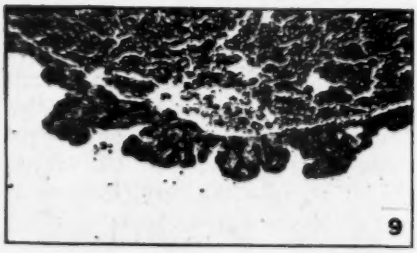
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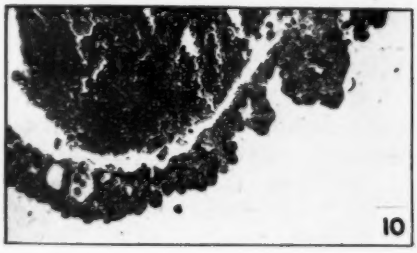
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Atypical Growth

Three points are clear from the experiments: (1) Since the abnormalities are induced by treatment of either egg or sperm and since the egg contains many times as much cytoplasm as does the sperm, it is obvious that the amount of cytoplasm present at the time of treatment makes little difference as far as the abnormalities are concerned. This fact alone leads to the presumption that the cell changes responsible for developmental abnormalities must take place in the nucleus. However, from other evidence this impression can be fully substantiated. It will be recalled that in the case of gynogenetic development, the amount of abnormality was slight despite the fact that very large doses of radiation had been applied to the sperm before fertilization. Hence, since the degree of injury varies with the dose of radiation only so long as the sperm nucleus is functional, it is clear that the main irradiation injury resulted from changes induced in the nucleus by the radiation.

(2) The changes induced were caused by a single application of the affecting agent. Thus, whereas there are on record numerous examples of gradual or adaptive cell change, as for example the change from normal to precancerous to cancerous condition following applications of a chemical carcinogen, here is a cell change occurring during the brief interval of exposure to radiation, which leads to a vastly altered pattern of growth—possibly even to malignant growth. Hence, the matter of sudden cell change must be kept in mind as a possibility along with the gradual and adaptive changes. It should be clear, however, that the evidence presented here is in no way antagonistic to the idea of gradual or adaptive change and that at this time there is no reason for excluding either possibility.

(3) Since the sperm cell at the time of treatment was free-living, it is evident that the growth abnormalities resulted from damage to a single cell, in fact to a haploid cell. From this it follows that the presence of a normal haploid nucleus (egg nucleus in this case) is not sufficient to insure normal growth when an injured haploid nucleus exists in the zygote. This fact does not correlate well with the classic view that genetic factors of normalcy are dominant.

Biologic Action of Radiation

Previously the author (*15*) developed the generalization that control activities of the cell, those depending on substances present in very limited amounts (e. g., genic), may be affected by relatively small amounts of radiation, whereas functional activities, those depending on varying amounts of substrate materials, etc., require much larger amounts to cause significant change. The observation that the amount of radiation required to stop sperm motility is many orders of magnitude greater than that required to produce growth abnormalities strongly bears out this general view. The findings, therefore, fit well in a general picture and emphasize again the importance of giving attention to the critical concentration of substances affected when considering either the factors controlling radiosensitivity or the mechanism of action of high energy radiations on cells.

Germ Cell Injury

It is obvious from the results that an extensive amount of first-generation injury may be caused by exposure of mature sperm to X-rays. Whether similar changes are induced in mammalian forms including man is, of course, not known. However, since the fertilization process is so widely prevalent and so generally similar throughout the living world, it seems logical to expect similar abnormalities in the higher forms as well. Such abnormali-

ties would not be seen as often in offspring of higher forms, however, since abnormal embryos are usually eliminated by absorption or abortion.

CONCLUSIONS

Evidence has been obtained indicating that abnormal tissue growth in the frog may be induced by exposure of single cells to single short treatments with X-rays. In obtaining this evidence, advantage was taken of the free-living character of the sperm cell and the fact that this cell (in conjunction with the egg) gives rise rapidly to tissues in which the abnormal behavior becomes manifest.

The changes responsible for growth lesions apparently occur in the nucleus of the irradiated cell. This is indicated by (*1*) the fact that the sperm nucleus after heavy irradiation will enter the egg (as part of the sperm cell) but will not fuse with the egg nucleus, and (*2*) the fact that growth abnormalities are not obtained in those cases in which the sperm nucleus is not functional. It therefore seems clear that the growth abnormalities do not occur when the irradiated sperm nucleus fails to take part in subsequent nuclear activity.

Furthermore, the fact that the sperm cell was free-living at the time of treatment, that it has a haploid number of chromosomes, and that a normal egg fertilized with a treated sperm will give rise to growth abnormalities in later development, makes it clear that growth modifications may result from injury to only half of the chromosome complement of the cell.

These findings, therefore, may be regarded as evidence supporting the view that a radically changed pattern of multicellular growth may be set in motion by the sudden modification of nuclear substances in a single cell.

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Tumor Enzymology

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INTRODUCTION

The metabolism of living tissues is carried on by a diverse and intricate mosaic of enzymic catalysis. Under normal conditions and over the greater part of the life of the host, each tissue maintains a steady and consistent enzymic pattern. Under abnormal circumstances, this pattern may be altered by injury to one or more of the component enzymes. Thus, under conditions of dietary depletion in vitamins (*1*, *2*) or essential metals (*3*, *4*), the activity of certain tissue enzymes of the host is impaired. The introduction of toxic agents into the host may also result in enzyme impairment, such as the effect of carbon monoxide inhalation on respiratory activity. When a tissue becomes neoplastic, a change in one or more of the enzymes may occur. For present purposes, it is relatively unimportant to know whether such a change, if it does occur, is the cause of the neoplasia, the consequence of the neoplasia, or the result of processes external to the affected tissue. All that we wish to establish at the present time is whether a change in enzymatic activity

is the specific accompaniment of neoplasia and to what extent this change occurs.

The possibility of the study of such changes in highly active and available systems has led to a large number of studies by numerous investigators. It would not be unfair to say that, with the exception of the work of Warburg and that of some others, few fields of endeavor have presented such a chaotic mass of isolated, contradictory, and unrelated data as has tumor enzymology. Too often, work in this field has appeared to be incidental to the spare time of the busy clinician or of the occasionally curious biochemist. It is only fair to point out that adequate material for the study of experimental cancer has only comparatively recently been made available. Much of the earlier work in tumor enzymology may be summarized by saying that most tumors appear to have qualitatively the same kind of enzymes as normal tissues have. This conclusion, based on the study of unrelated tumors and unrelated normal tissues, is not unimportant, but it adds little to the elucidation of the problem of cancer. It is instructive to assess the contribution of Warburg to this problem, because the importance of the use of the best available material and the employment of appropriate experimental conditions have perhaps never been better illustrated (*5*). Warburg first developed the most appropriate experimental methods for his studies, then systematically utilized a variety of tissues, reported data on many samples of each tissue under different conditions, and most important of all centered his attention on the problem of cancer. These were self-evident approaches and methods, but they were lacking in most of the work in this field. Many of Warburg's interpretations and conclusions have subsequently been modified by later workers, but his approach to the subject may serve as a model.

Warburg's work may be said to have completed the second phase of tumor enzymology, of which the scattered work of the pioneer investigators may be said to have been the first. The third, or contemporary phase, may be considered as differing fundamentally from the first two in laying emphasis on the study of homologous pairs of tumors and their normal tissues of origin. The desirability of such a development in this field was first indicated by Voegtlin (*6*) in a comprehensive review of the biochemistry of cancer published in 1937. Voegtlin emphasized the value of such comparative biochemical studies, the care required in the selection of tumors and their normal tissues of origin, the consideration due to individual variations and to dietary factors, and the desirability of limiting studies to neoplasms of known histogenesis in which the malignant cells are highly concentrated. Voegtlin's views heralded the extensive development in this country of the biochemical approach to the cancer problem in which tumor enzymology has shared the general advance (*7*).

The development of the third phase of tumor enzymology has had to wait for the preparation of suitable carcinogenic agents and for the development of more or less pure strains of animals. It is an illustration of the increasingly greater interdependence of various workers in this field. By the use of homologous normal and neoplastic tissues, e. g., liver and hepatomas, lymph nodes and lymphomas, etc., it is possible to approximate our present goal, namely, to observe whether the enzymes of a given normal tissue change in activity when the tissue becomes neoplastic. Such comparative studies are a truly valid contribution to the problem of cancer, whereas earlier comparisons of

such unrelated tissues as the Jensen sarcoma and rat liver, important though they may be, are of interest chiefly to the field of comparative biochemistry. Transplantation of growing neoplastic tissue in animals affords an excellent method of obtaining almost pure material under readily controllable conditions, and this method should be used where possible.

Running simultaneously through the three consecutive phases of tumor enzymology has been the desire of a few investigators to see whether the presence of a tumor affects the enzymes of tissues far removed from the site of the tumor. This represents the attempt to observe a systemic effect elicited in the tumor-bearing animal, which, apart from its intrinsic interest, might lead to the development of a clinical diagnostic test for cancer. Thus, the enzymes of the livers, kidneys, serum, etc., of normal and of tumor-bearing human beings and animals have been compared. This approach is of potential practical value, but it obviously requires the careful distinguishing of any effect observed with tumors from those which may be elicited by other pathologic conditions.

Work in the field of tumor enzymology should consider the characterization of tumors in terms of enzymatic activity, not the enzyme in terms of the tissues in which it occurs. The latter characterization is important but secondary to the main purpose of this field. In most reviews on the subject of tumor enzymology, including the latest one of Köhler (*8*), the subject headings are under the names of individual enzymes. As a result a great many tumors are separately described under a number of different enzymes, and a clear-cut analysis of the state of any one tumor is never assembled.

If emphasis is, therefore, to be placed on the tumor, the tumor must be considered against its background of pathology and must be independently characterized by histologic criteria. As new tumors are developed, the necessity for histologic control becomes imperative. There is, furthermore, the possibility of alteration in the type of certain transplanted tumors during serial transplantation. Although primary emphasis is laid upon the tumor and not upon the individual enzyme, it is clear that the results obtained will be no better than the technique employed in measuring enzymatic activity.

In this review emphasis is laid on comparisons on the one hand of neoplasms and their normal tissues of origin, and on the other of similar tissues in normal and in tumor-bearing animals. Each tissue is characterized by the activity of several enzyme systems or by the concentration of several organic and inorganic components. The discussion centers chiefly on the activity of individual, relatively well defined enzymes rather than on factors such as tissue respiration and glycolysis, which involve complexes of concurrent enzymic activities. No attempt is made to give anything more than a comprehensive summary of only those enzyme data that appear to be relevant to the cancer problem.

COMPARISON OF THE ENZYMATIC ACTIVITY OF TUMORS WITH THAT OF THE NORMAL TISSUE OF ORIGIN

The data in table 1, as well as those in succeeding tables, are essentially comparative, and in every case reported the comparison had been made by the same investigator or groups of investigators. The comparisons have thus been made throughout under the same conditions. Under the headings of the columns in table 1 entitled "Rat hepatomas" and "Mouse hepatomas" appear those tumors which either

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arose spontaneously or were induced by the indicated carcinogen. All of the mouse hepatomas were transplants.

Hepatomas and Liver

Few tissues lend themselves so admirably to comparative studies as do the liver and hepatomas. Hepatomas arise in liver cells of different species and in various animal strains either spontaneously or by induction through the administration of chemical agents. The liver contains a wide assortment of highly active enzymes. The possibility therefore exists of studying alterations in a number of biocatalytic systems in hepatomas of different origin and in various strains and species of animals. Further comparison with growing hepatic tissues is afforded by the study of fetal and of regenerating liver in which, in contrast with the case of the hepatomas, the growth of the tissue is controlled.

The hepatomas include those that were induced by the inclusion of *p*-dimethylaminoazobenzene or of 2-amino-5-azotoluene in the diet of rats, and those that arose spontaneously in mice or were induced by the injection of 2-amino-5-azotoluene or of carbon tetrachloride. The rat hepatomas were first developed in Japan; transplantation of the aminoazotoluene-induced tumors was accomplished by Iikubo. (See Shear (*9*) for review.) Transplantation of *p*-dimethylaminoazobenzene-induced hepatomas was accomplished by White (*10*) at the National Cancer Institute. The chemical studies on the mouse hepatomas were performed on tumors developed at the Institute by Andervont and by Edwards. A recent paper by Andervont, Grady, and Edwards (*11*) summarizes the histologic characteristics of many of the mouse hepatomas.

This section includes all of the available data on the enzymes and certain other components of the hepatomas and of related normal hepatic tissues. Some of the data are on primary tumors, others on transplants. These will be distinguished. Where comparison of the same enzyme in the primary tumor and in the transplant was possible, it was often found that the activity in the primary hepatoma was intermediate in value between that of the normal liver and that of the transplant. This is probably due in part to the presence of normal liver cells in the primary hepatoma.

The fact that the data are derived from the results of many investigators who used different experimental methods raises the problem of how to present them most effectively. The details of the various determinations and the methods are available in the original articles to which reference may be made. The data are assembled and compared on the following basis: the value for the activity of enzyme in normal adult liver, irrespective of species or strain, is arbitrarily set at 100. The corresponding values of the enzymes in the hepatomas, or fetal or regenerating liver are then given on a comparative basis and are less or greater than 100 depending upon whether the activity of the enzyme concerned is respectively less or greater than that in normal liver. The values thus represent the fractional part of the activity of normal liver and are based on data obtained from the literature. The data are given in table 1.

Table 1 reveals on the whole striking differences in the activity of regenerating, of fetal, and of neoplastic liver tissues. With two exceptions, xanthine dehydrogenase activity and the content of total solids, regenerating rat liver is very similar in all respects to normal adult (resting) liver. The activity of amylase in fetal liver is of the same order as that of adult liver, but the activity or concentration of every other component studied

TABLE 1.—*Relative activity of enzymes or concentrations of components in hepatomas, fetal, and regenerating liver* [1]

Enzyme or component	Rat hepatomas induced with—				Mouse hepatomas [2]						Fetal liver			Regenerating rat liver
	p-Dimethylaminoazobenzene		Aminoazotoluene		Aminoazotoluene-induced in—			Spontaneous		Carbon tetrachloride-induced				
	Transplanted	Primary	Transplanted	Primary	I mice	C mice	C3H mice	C3H mice	A mice	A mice	Rabbit	Rat	Cat	
Arginase	10 (12)	14 (13)			16 (12)		12 (12)	12 (12)	13 (12)	14 (12)	35 (12)			100 (12)
Do		27 (14)												
Catalase	1 (12)				18 (12)		3 (12)	4 (12)	11 (12)	36 (12)	10 (12)			100 (12)
Xanthine dehydrogenase	50 (12)				100 (12)		26 (12)	28 (12)	50 (12)	60 (12)	0 (12)			50 (12)
d-Amino acid oxidase	12 (15)											12 (15)		91 (15)
Riboflavin	16 (16)	19 (17)		63 (18)	39 (16)		39 (16)	42 (16)	42 (16)	47 (16)		19 (16)		100 (16)
Do		20 (19)										16 (17)		
Coenzyme I		11 (18)										[3] 22 (20)		
Cytochrome oxidase	28 (15)	33 (15)			33 (15)		33 (15)	33 (15)	33 (15)	33 (15)		33 (15)		100 (15)
Cytochrome c		22 (21)												
Succinic oxidase						14 (22)								
Histidase		5 (13)												
Thymonucleodepolymerase	78 (12)				78 (12)		150 (12)	143 (12)	110 (12)	115 (12)	100 (12)			100 (12)
Acid phosphatase	200 (12)				83 (12)		175 (12)	183 (12)	175 (12)	183 (12)	64 (12)			112 (12)
Alkaline phosphatase	13,550 (12)				25 (12)		0 (12)	25 (12)	125 (12)	100 (12)	0 (12)			100 (12)
Amylase [4]	100 (12)				100 (12)		138 (12)	100 (12)	85 (12)		100 (12)			100 (12)
Glycogen	0 (23)		12 (24)		[5] + (25)		[5] + (25)	[5] + (26)	[5] + (25)	[5] + (25)				
Glucose			30 (24)											
Lactic acid			200 (24)											
Proteinase	200 (27)		30 (28)											100 (27)
Dipeptidase			100 (28)											
Tripeptidase			100 (28)											
Ascorbic acid		62 (13)	220 (29)											
Glutathione	77 (30)	89 (31)	142 (29)				98 (30)	98 (30)	100 (30)		0 (30)			100 (30)
Transaminase		33 (32)											25 (33)	92 (33)
Urea synthesis	0 (34)													
Urea	200 (14)	150 (14)			200 (14)		200 (14)				200 (14)			100 (14)
Nonprotein nitrogen			100 (35)											
Amino nitrogen			100 (35)											
Creatine	100 (36)		100 (35)						100 (36)	100 (36)				
Creatinine	100 (36)		100 (35)						100 (36)	100 (36)				
Phosphatide			57 (37)											
Fatty acids			35 (37)											
Free cholesterol		143 (13)	127 (37)											
Esterified cholesterol		310 (13)	150 (37)											
Acid-soluble phosphorus			110 (37)											
Acid-insoluble phosphorus			65 (37)											

[1] Values for normal adult liver taken as 100, irrespective of species or strain. Figures in the table refer to the relative activity of each enzyme or concentration of each component on this basis. References are in parentheses.

[2] Transplanted hepatomas.

[3] Coenzymes I and II.

[4] Probably owing to 2 or more separate enzymes.

[5] Histologic evidence.

TABLE 1.—*Relative activity of enzymes or concentrations of components in hepatomas, fetal, and regenerating liver*—Continued

Enzyme or component	Rat hepatomas induced with—				Mouse hepatomas						Fetal liver			Regenerating rat liver
	p-Dimethylaminoazobenzene		Aminoazotoluene		Aminoazotoluene-induced in—			Spontaneous		Carbon tetrachloride-induced				
	Transplanted	Primary	Transplanted	Primary	I mice	C mice	C3H mice	C3H mice	A mice	A mice	Rabbit	Rat	Cat	
Vitamin A				0 (*38*)										
Biotin		45 (*39*)			30 (*39*)							27 (*39*)		100 (*39*)
Copper	80 (*40*)				60 (40)			80 (*40*)						100 (*40*)
Total solids	59 (*16*)		63 (*41*)		78 (*16*)		78 (*16*)	83 (*16*)	83 (*16*)	72 (*16*)		75 (*16*)		81 (*16*)
Total nitrogen			116 (*41*)											
Total phosphorus			125 (*41*)											
Total sulfur			124 (*41*)											
Sodium			163 (*42*)											
Potassium			487 (*42*)											
Calcium			61 (*42*)											
Magnesium			191 (*42*)											
Iron			66 (*42*)											
Iodine			100 (*42*)											
Chlorine			177 (*42*)											
Sodium chloride			177 (*42*)											
Ash			135 (*42*)											

in the former is invariably less than that found in the latter tissue. In the hepatomas, the activity of the enzymes are less than, equal to, or greater than that of the corresponding enzymes in normal adult liver. With a few exceptions, most of the enzymes are diminished in the hepatomas as compared with normal liver and so resemble the comparison of fetal with adult liver. The tissue components which are apparently more concentrated in the liver than in other body tissues, e. g., arginase, catalase, riboflavin, etc., are considerably diminished when the liver becomes neoplastic. It is possible that many of the hepatic enzymes and other components are simply stored in the liver, and the capacity (or necessity) for storage is lost when the liver becomes neoplastic. On the whole, however, it might be said that, enzymatically, fetal liver resembles the hepatomas more than it does either adult or regenerating liver. Burk (*43*) has shown that the glycolytic power of regenerating rat liver is very similar to that of normal adult liver, but that the glycolytic power of fetal liver like that of the hepatomas is considerably greater than that of adult liver. Fetal liver contains a large amount of hematopoietic elements, and hence the results obtained with this tissue must be accepted with some reserve.

The comparison of such obviously separate tissues as adult, fetal, and regenerating liver and hepatomas offers the metabolic picture of tissues at definitely separable states. The data are clearly distinguishable on the basis of the state of the tissue. It would be advantageous, however, to have more data on the intermediate stages involved, such as the change of liver into the neoplasm, the course of regenerating liver, and the growth of fetal into adult tissue. A few studies of the latter two changes are available (*5*, *14*, *39*, *44*) and at least one study of the former (*32*). A dynamic picture is needed of the changes involved, and this is obviously the most immediate task.

After this consideration of the general types of hepatic tissue, attention can be given to the comparison of the hepatomas in each of the two species, rats and mice, in which these tumors have been studied. Hepatomas in rats have been induced by the feeding of two different carcinogenic chemicals. Most of the biochemical data on the tumors induced with *p*-dimethylaminoazobenzene have been reported by American workers, those on the aminoazotoluene-induced tumors by the Japanese. Unfortunately, only a few reports of studies on the same chemical system in both hepatomas are available. For mice, however, there are considerable data on many enzyme systems, all of which were studied in transplanted hepatomas of different origin in several inbred strains (*12*). With these studies in mice supplemented by the few in rats, it has been possible to observe what effect the mode of origin of the tumor and the animal strain in which it arises have upon the enzymatic properties of the tumor.

Arginase and Urea

Arginase, the enzyme which catalyzes the hydrolysis of arginine to ornithine and urea, is diminished to approximately the same extent in hepatomas in rats and in various strains of mice. The results on the *p*-dimethylaminoazobenzene-induced hepatomas in rats from two different laboratories are similar. The arginase activity of the rat hepatomas and of the hepatomas of diverse origin in several strains of mice is very nearly the same. The content of urea in the hepatomas and in fetal liver is twice as great as normal. The urea in these tissues is probably due to the action

of the arginase on arginine either derived from tissue protein breakdown or brought to the tissue by the blood stream. The urea-synthesizing mechanism, described by Krebs for normal adult rat liver (*45*), is apparently missing in the rat hepatoma (*34*). The arginase component of the mechanism, although diminished, is still present; it is the synthetic portion of this cycle, e. g., the portion responsible for the synthesis of arginine from citrulline or ornithine and ammonia that is deficient. A specific function of the normal liver thus apparently vanishes when the liver becomes neoplastic.

When ammonia is added to slices of liver or of hepatoma suspended in a glucose-containing medium, the rate of disappearance of the ammonia is distinctly greater in the former than in the latter tissue (*34*). The ammonia consumed is presumed to enter into a synthetic reaction with the glucose or its conversion products. In view of the rapid growth characteristic of the hepatoma, it is somewhat surprising to find that the ammonia consumption is less than that in the normal liver, but it is possible that the synthetic processes of the tumor require other factors and perhaps other conditions than those employed.

Catalase, Cytochrome Oxidase, and Cytochrome c

The iron-containing components of normal liver, catalase, cytochrome oxidase, and cytochrome *c*, are considerably reduced when the liver becomes neoplastic. The exact physiologic function of catalase is not known; it is identified by its high rate of splitting of hydrogen peroxide. The lack of evidence for the presence of the latter substrate in animal tissues has been the cause of the inability to assign catalase a definite and known function. Nevertheless, it appears to be an invariable component of the livers (and kidney and erythrocytes) of all mammals. Cytochrome oxidase is a participant in the respiration cycle in all tissues, and its substrate is the readily identifiable tissue component, cytochrome (*46*). Of the various cytochromes in tissues, only cytochrome *c* has been isolated. For the synthesis of catalase and of cytochrome oxidase by the tissues, a definite level of copper in the diet of the host animal has been found necessary (*3, 4*). In this connection it is interesting to note that the copper content of hepatomas is lower than that of normal liver.

Catalase activity in the rat hepatoma is reduced to an almost negligible amount as compared with that of normal liver. The reduction is not so marked in the mouse as in the rat hepatomas, but here, too, the activity is much less than normal and appears to be slightly related to the strain of mouse in which the hepatoma appears. The activity of catalase in the induced and in the spontaneous hepatoma in C3H mice is very nearly the same, but the activity of the induced and spontaneous hepatomas in A mice is quite different in the two types of tumor and different also from that in the C3H mice. The cytochrome oxidase activity is very nearly the same for hepatomas in all the mice strains.

The catalase (and arginase) activity of the induced tumor in I mice is the same as that found in the same tumor transplanted to F_1 hybrids of this strain with C3H and with dilute brown mice (*12*). Hybridization, at least for the first generation, does not affect the enzymic properties of the tumor. Furthermore, in any one hepatoma in a given mouse strain the enzymic activity is invariably independent of the age or the growth rate of the tumor.

Both cytochrome oxidase and cytochrome *c* are reduced to nearly the same extent in the rat hepatoma. This reduction is of some interest when it is considered that the respiration (in which these

components participate) is practically the same in the hepatoma and in normal and regenerating liver (*43*). Shack (*15*) has pointed out that the liver, whether resting or regenerating, has, in contrast with the hepatoma, a considerable reserve of the oxidase - cytochrome system. When *p*-phenylenediamine in addition to glucose was added to liver and to hepatoma, the former tissue responded by a greater oxygen consumption than did the latter (*22*). Since the hepatomas do not possess an oxidative reserve, they can respond to demands for more energy by the supply of substrates only by an increase in glycolysis, whereas normal and regenerating liver (the latter like hepatoma is also rapidly growing) may respond by increased oxidation (*15*). The oxygen consumption of the hepatoma is thus limited by the cytochrome system. This concept assists in the general interpretation of the high glycolysis of most tumors whose cytochrome oxidase values fall within the range of the hepatomas.

Xanthine Dehydrogenase, d-*Amino Acid Oxidase*, *Succinic Oxidase*, *Riboflavin*, *Thiamin*, *Coenzyme I*, *Vitamin A*, *and Biotin*

Xanthine dehydrogenase, *d*-amino acid oxidase, succinic oxidase, riboflavin, thiamin, coenzyme I, vitamin A, and biotin either have in common a known riboflavin structure or require riboflavin in the diet for their proper level of function (*1*, *2*, *47*). With one exception, xanthine dehydrogenase is present in greatly diminished activity in the hepatomas as compared with normal liver. The value for the hepatoma in the I strain is the same as that of normal liver. The drop in the two hepatomas of the C3H strain is very nearly the same and the drop in the two hepatomas in the A strain is very nearly the same but different from that in the C3H strain. The riboflavin content of all the mouse hepatomas, however, is very nearly the same and is considerably lower than the value for normal liver. Since xanthine dehydrogenase, *d*-amino acid oxidase, and succinic oxidase are flavin enzymes, it is not surprising to find their activity reduced in the riboflavin-low hepatomas but it is equally clear that there is no exact correlation between the drop in riboflavin content in the tumor and the drop in either amino acid oxidase or xanthine dehydrogenase activity. Shack (*15*) suggested that the lowering of the *d*-amino acid oxidase activity may be owing to a deficiency in the protein component as well as in the flavin prosthetic group.

The oxidation chain in tissues is presumed to involve the action of molecular oxygen on cytochrome oxidase previously reduced by the reduced form of cytochrome. The latter in turn is reduced by the flavoprotein enzymes. The iron and flavin systems are thus closely linked in the respiration cycle of animal tissues. However, although both cytochrome oxidase and *d*-amino acid oxidase are reduced in activity when the liver becomes neoplastic, the latter is reduced to a much greater extent. The fall in activity of the amino acid oxidase is also much greater than that of xanthine dehydrogenase. Since the last two enzymes are flavoproteins and there appears to be more than enough flavin in the tumor to account for their combined activity, the unequal fall in their activity would lend weight to the suggestion that the fall is caused by changes in the protein moiety of these enzymes. In any event, the change of liver cells from normal to neoplastic is accompanied by an unequal change in these as in many other enzyme systems.

Coenzyme I, which participates in the

oxidation mechanism, contains nicotinic acid amide as a component and hence is also related to the vitamins. The content of coenzyme I and vitamin A in the hepatoma is considerably lower, that of thiamin only slightly lower, than that, respectively, in normal liver. The biotin values for the rat and mouse hepatomas were considerably lower than the corresponding values for the normal livers of these species. The lowered value in the case of the mouse hepatoma was characteristic only of the later growth of the tumor. A single specimen of a human liver carcinoma gave in general the same picture as that of the rat hepatoma.

Amylase, Glycogen, Glucose, and Lactic Acid

Amylase, which is probably composed of at least two separate enzymes (*48*), acts upon glycogen with the liberation of reducing groups. The activity of this enzyme system in the hepatomas differs very little from that in normal liver. Here is at least one system in liver which is not obviously affected when the liver becomes neoplastic. This is all the more interesting in view of the observation that the rat hepatoma is nearly devoid of glycogen. The mouse hepatomas on the other hand all contain glycogen in quantities which at present are not known. The amylase system is retained in the hepatoma independently of the capacity of the latter to store glycogen. Orr and Stickland (*49*) have pointed out that the substrate for glycolysis in the liver is glycogen, in the rat hepatoma it is glucose. Glycogen may be unnecessary in the hepatoma, but if so the reason for the presence of the amylase system becomes obscure. The relative proportion of glucose in the rat hepatoma is low, that of lactic acid is high, as compared with that of the normal liver, and this in view of the greater glycolysis in the former tissue is not surprising.

Peptidases, Transaminase, and Histidase

The proteolytic activity of the *p*-dimethylaminoazobenzene-induced hepatoma in the rat is considerably greater than that of normal liver. The substrate used for these studies was hemoglobin. The proteolytic activity of the aminoazotoluene-induced rat hepatomas, when casein and gelatin were used as substrates, was found to be much less than that of normal liver. Proteolysis with the former tumor was accelerated by addition of cysteine, but with the latter tumor it was unaffected by the addition of sulfhydryl. Although different conditions of extraction of the tissues were employed and different substrates were used, it is not believed that they were primarily responsible for the difference in the relative proteolytic activity shown. Reference to data on other enzymes in table 1, which were obtained with mouse tumors induced by different carcinogens in different strains, shows clearly that quite opposite results even under the same conditions on the same enzyme in tumors in different strains are not exceptional, e. g., thymonucleodepolymerase and alkaline phosphatase. The difference in proteolytic activity in the two hepatomas may well be ascribed to differences in the animal strains. The activity of dipeptidase and tripeptidase in the aminoazotoluene-induced rat hepatoma is the same as that of normal liver. It is surprising that the distribution in the enzyme sequence in the breakdown of the proteins should be so unequal when the liver becomes neoplastic. As in the case of the amylase system discussed previously, the reason for the retention or loss of catalytic systems appears to be obscure.

Tumor growth involves the continuous synthesis of protein by the peptidases. This synthesis must be coupled with energy-producing mechanisms, since the

equilibrium between protein and peptides or amino acids lies far on the side of hydrolysis. In view of the relatively low proportion of the oxygen-utilizing systems in the hepatomas and in fetal liver, the energy for synthesis in the tissues must be largely supplied by glycolysis. Burk (*43*) has shown that glycolysis is high in these tissues. The problem of relating biochemical synthesis to energy-producing mechanisms offers probably the most fundamental task in the elucidation of the neoplastic process.

Histidase, an enzyme which so far has been found only in liver and which splits ammonia from the imidazole ring of histidine, is almost nonexistent in the rat hepatoma—another example of a specific hepatic function which practically disappears when the liver becomes neoplastic. Transaminase, which is concerned with the transfer of the amino group from certain amino acids to keto acids, decreases progressively in the liver when a hepatoma is induced, and finally reaches a low level characteristic of the hepatoma (*32*).

Thymonucleodepolymerase and Phosphatase

The activity of the thymonucleodepolymerase, which is followed by the progressive depolymerization of sodium thymonucleate, is not very greatly changed in hepatomas as compared with normal liver. It is slightly below normal in the rat and in one of the mouse hepatomas and slightly greater than normal in the rest of the mouse hepatomas. This system, like amylase and certain of the peptidases, is practically completely retained when the liver becomes neoplastic.

The activity of acid phosphatase, with one exception, is significantly increased in both rat and mouse hepatomas. In *p*-dimethylaminoazobenzene-induced rat hepatoma the augmentation of alkaline phosphatase activity is phenomenal, amounting to nearly 150 times that of the normal liver. It may be that this is brought about by the inclusion of bone elements in the tumor. The alkaline phosphatase activity of the mouse hepatomas varies from values lower than that of normal liver to values that are little different. With this enzyme there appears to be a strain difference in mice similar to the case of xanthine dehydrogenase and of catalase. The activity in hepatomas in the C3H strain is lower than normal, that in hepatomas in the A strain is nearly the same as normal. The lack of correlation in each of the tumors of the relative values for thymonucleodepolymerase and for phosphatase indicates that the two enzymes are different. The macro determinations of phosphatase in the hepatomas have been independently confirmed by histologic determinations (*50*).

Since the thymonucleodepolymerase acts upon an exclusively nuclear substrate, this enzyme may be assumed to exist in the nucleus. Nuclear activity in adult, in fetal, in regenerating, and in neoplastic hepatic tissue, insofar as the state of nucleic acid is concerned, is practically the same. The activity of the factor responsible for the state of thymonucleic acid in the nucleus is independent of the rate of hepatic tissue growth. The proportion of desoxynucleoprotein in rat liver and in transplanted rat hepatoma, as well as the analytical composition and physical properties of the preparations from each of these tissues, is practically the same (*30*). It is possible that differences between liver and hepatoma must be sought for rather in the cytoplasmic components of these tissues.

Phosphatide, Fatty Acids, and Cholesterol

The content of phosphatides and of fatty acids in the rat hepatomas is lower than in normal liver but that of both free and es-

terified cholesterol is distinctly greater. These results are the same for hepatomas induced with either *p*-dimethylaminoazobenzene or aminoazotoluene. That a qualitative as well as a quantitative difference in the hepatoma cholesterol may exist is indicated by the report of the presence of 7-dehydro-cholesterol in the cholesterol fractions of the hepatoma (*51*).

Ascorbic Acid and Glutathione

The analysis of tissues for ascorbic acid and glutathione is beset by many technical difficulties, and the data must be accepted with some reserve. The ascorbic acid content and the glutathione content of the hepatoma in rats induced with *p*-dimethylaminoazobenzene have been reported to be lower and those in the rat hepatomas induced with aminoazotoluene to be distinctly higher than the corresponding values for these components in normal rat liver. On the other hand, the glutathione content of the mouse tumors investigated was about the same as that of normal mouse liver. The sulfhydryl groups of normal liver, available to titration after denaturation of the liver proteins, are greater in number per gram of tissue than the corresponding groups in the proteins of the hepatoma (*30*). This is interesting in view of the higher sulfur content of the hepatoma, but the difference may be explained on the basis that more of the sulfur in the hepatoma may be in the form of disulfide rather than sulfhydryl groups.

Comparison of the ascorbic acid plus glutathione content of the two kinds of rat hepatoma reveals the greater reducing environment that must exist in the hepatomas induced by aminoazotoluene. It is possible that the failure to cause increased proteolysis in extracts of these hepatomas by addition of reducing agents may have been ascribable to the presence of already maximal quantities of these agents.

Nitrogen and Creatine

The content of nonprotein nitrogen, of amino nitrogen, and of creatine and creatinine is practically the same in hepatomas as in normal liver. Creatine is formed by the interaction of choline, methionine, glycine, and arginine (*52*, *53*). The amidination of glycine may occur in the kidney, but the methylation of guanidoacetic acid occurs in the liver (*54*). The capacity to produce creatine (and creatinine), which represents a phase of amino acid metabolism, is yet another system that is apparently completely retained when the liver becomes neoplastic.

Total Solids and Metals

The proportion of water in the hepatomas irrespective of origin, strain, or species of animal appears to be distinctly higher than in normal liver. If this water is mainly extracellular, it might be expected that the sodium- and chloride-ion concentrations would also be higher in the tumors. This is indeed the case. The proportion of potassium in the tumor is phenomenal. Iron is lower in the tumor than in normal liver, and this is in keeping with the finding of decreased iron-containing enzyme systems in the hepatomas.

Lymphomas and Lymph Nodes

In table 2 are the data obtained on a transplanted lymphoma in A mice and the normal lymph nodes used as controls.

TABLE 2.—*Transplanted, subcutaneous lymphoma and lymph nodes in strain A mice* [1]

Enzyme	Lymphoma	Reference
Arginase	130	(*14*)
Urea	20	(*14*)
Catalase	50	(*55*)
Xanthine dehydrogenase	960	(*55*)
Thymonucleodepolymerase	[2] 1	(*56*)
Acid phosphatase	20	(*57*)
Alkaline phosphatase	125	(*57*)

[1] For explanation of figures, see table 1, footnote 1.
[2] Approximate.

A brief histologic description of this tumor has been given (*1A*).

Normal lymph nodes contain the greatest amount of thymonucleodepolymerase activity of all the animal tissues studied. This might be related to the relative size of the nucleus as compared with the cytoplasm in lymph nodes. Since thymonucleic acid exists almost wholly in the nucleus, it might be expected that the enzyme or enzymes that affect this acid would be high in activity in highly nuclear material. When the nodes become neoplastic, however, the thymonucleodepolymerase activity drops to a very low value despite the fact that the relative size of the nucleus remains fairly large in the tumor. The interrelation of morphology and physiologic function is still obscure.

The activity of arginase and alkaline phosphatase increases slightly in the lymphoma, that of xanthine dehydrogenase increases considerably, whereas that of acid phosphatase definitely decreases.

Mammary Tumors and Breast

The only data in the literature useful for comparative purposes are those obtained on spontaneous mammary tumors in mice. The control tissue was either hyperplastic breast induced by subcutaneous injection of stilbestrol or lactating breast. The data are given in table 3.

Hyperplastic breast is not an altogether unobjectionable tissue for comparison with the spontaneous mammary tumors, and hence the comparisons made must be accepted with some reserve. Examination of the data (table 3) reveals that the enzyme activity in the tumor is either equal to or distinctly greater than that in the hyperplastic breast. The activity of the various enzymes studied in mammary tumors arising spontaneously in different strains is the same and indicates the absence of any effect of the strain on the tumor enzyme. This apparent fact differentiates the spontaneous mammary tumors to some extent from the transplanted hepatomas in mice (table 1).

Table 3.—*Mammary tumors and breast in mice of strains C3H and A* [1]

Strain of mouse	Enzyme	Spontaneous mammary tumors	Reference
C3H	Arginase	[2] 170	(*14*)
A	do	[2] 173	(*14*)
C3H	do	[3] 248	(*14*)
C3H	Catalase	[2] 100	(*55*)
C3H	do	[2] 100	(*55*)
C3H	Xanthine dehydrogenase	[2] 150	(*55*)
C3H	Thymonucleodepolymerase	[2] 100	(*56*)
C3H	Acid phosphatase	[2] 116	(*57*)
A	do	[2] 111	(*57*)
C3H	Alkaline phosphatase	[2] 244	(*57*)
A	do	[2] 222	(*57*)

[1] For explanation of figures, see table 1, footnote 1.
[2] Hyperplastic breast induced by stilbestrol as control tissue.
[3] Lactating breast as control tissue.

Rhabdomyosarcoma and Muscle

In the literature there is a single reference to work in which an enzyme system in a transplanted rhabdomyosarcoma and in normal muscle is studied (*22*). The enzyme used was succinic oxidase, and the relative activity of the transplanted rhabdomyosarcoma in this case was 17. The activity of the succinic oxidase was obviously greatly reduced in the rhabdomyosarcoma as compared with normal muscle.

Adenocarcinoma of the Stomach and Intestine and Normal Gastric and Intestinal Mucosa

Comparisons of the enzymatic activity of transplanted adenocarcinomas of the stomach and of the intestine in mice with the corresponding normal mucosa have been reported (*58*). The tumors were developed and described by Lorenz and Stewart (*59*, *60*). The enzyme data are given in table 4.

TABLE 4.—*Adenocarcinoma of the stomach and intestine and normal gastric and intestinal mucosa* [1]

Enzyme	Gastric adenocarcinoma [2]	Intestinal adenocarcinoma [2]
Pepsin	0 (58)	
Rennin	0 (58)	
Thymonucleodepolymerase	98 (58)	98 (58)
Acid phosphatase	59 (57)	56 (57)
Alkaline phosphatase	0 (57)	.1 (57)

[1] For explanation of figures, see table 1, footnote 1.
[2] Transplanted tumor in C3H mice.
[3] Transplanted tumor in A mice.

Pepsin and rennin are enzymes specific to the gastric mucosa, and apparently they vanish when gastric mucosa becomes neoplastic. The greatest activity of alkaline phosphatase for all body tissues is found in the small intestine, and this enzyme also apparently nearly disappears in the intestinal adenocarcinoma. The gastric adenocarcinoma contains little or no alkaline phosphatase, and in both types of adenocarcinoma the acid phosphatase is reduced to about half the activity of the normal mucosa. In contrast with these marked changes, the activity of thymonucleodepolymerase in the tumors is practically the same as in the respective normal tissues of origin.

Sugiura, Peck, and Stewart (*61*) in a report on the comparison of the tryptic, amylolytic, and lipolytic activity of a single primary human adenocarcinoma of the pancreas with normal human pancreas observed no difference in the activity of these enzymes. This is an interesting finding, but too great weight cannot be attached to observations on a single sample of tissue.

Carcinoma of the Prostate and Normal Prostate

Kutscher and Wolbergs (*62*) first demonstrated the presence of a highly active acid phosphatase (optimum pH 5.0) in normal prostate tissue, but the only comparison of this enzyme performed under the same conditions with carcinomatous prostate tissue is in the report by Gutman and Gutman (*63*). The relative activity of acid phosphatase at pH 5.06 of the tumor was 156.

The acid phosphatase in the carcinomatous prostate is definitely but not greatly increased over that of the normal tissue. No data appear to exist in relation to the alkaline phosphatase activity of the tumor, but Gutman, Sproul, and Gutman (*64*) and Woodard (*65*) have reported that the normal prostate contains very little of this enzyme. Of considerable clinical importance has been the discovery that the serum of patients with disseminated carcinoma of the prostate contains large amounts of the acid phosphatase (*63*).

Additional information concerning the phosphatase activity of prostatic carcinoma has been derived from extensive studies on the sites of osteoplastic bone metastases secondary to carcinoma of the prostate gland (*64*). Studies of the lumbar vertebrae and ribs of a patient with disseminated prostatic cancer revealed high acid phosphatase values and suggested that metastatic tumor cells arising from the tumor retain the high acid phosphatase activity of the primary cancer. It is necessary, however, to attempt to differentiate the activity of the phosphatase in the metastasis from that of the proliferating bone. Increased alkaline phosphatase activity is characteristic of growing bone in children, of hyperplastic bone (*65*), and of the osteoblastic type of osteogenic sarcoma (*66*). The bones studied in the case of disseminated prostatic carcinoma had not only a high acid but also a high alkaline phosphatase activity. Since the acid phosphatase activity of bones is very low, the presence of this enzyme at the site of the metastasis betrays the presence of prostatic tissue, but the high alkaline phosphatase activity at this site must in turn be

related in some fashion to the osteoplastic character of the prostatic metastasis. It seems strange that determinations of the phosphatase activity at pH 9 of the primary carcinoma of the prostate have not been reported, for if this value were known it might be possible to separate the activity of the skeletal metastasis from that of proliferating bone. It is interesting to consider in this connection the alkaline phosphatase values for the hepatomas in rats induced with *p*-dimethylaminoazobenzene (*10*, *57*). The normal liver has a very low alkaline phosphatase activity, but the hepatoma is extremely active in this enzyme. White and Edwards (*10*) in a description of the pathology of this tumor point out that the presence of this enzyme in considerable amount may be associated with the capacity for bone formation, and in one of the transplants of this tumor membranous bone formation was observed. Although the studies on the skeletal metastases of prostatic carcinoma have been highly interesting and suggestive, it is unfortunate that only skeletal metastases have been investigated. The bone itself, and particularly growing bone, is rich in alkaline phosphatase, and for this reason the results on this enzyme at sites of osteoplastic metastases may be confusing. Further studies on the primary carcinoma or on metastases to nonosseus tissues low in alkaline phosphatase would go far toward clearing up the problem.

Osteogenic Sarcoma and Bone

The chemical composition of bone has made extensive studies of the phosphatase mechanism in this tissue inevitable. The role of this enzyme in bone formation has been reviewed (*67*, *68*), and studies of the phosphatase activity in normal and neoplastic bone have been reported by several investigators [1] (*64*, *65*, *66*). Most of the work has been done with human tissues. Recently, an opportunity arose to study a transplantable osteogenic sarcoma in mice. The data are given in table 5.

Table 5.—*Osteogenic sarcoma and bone* [1]

Enzyme	Tumor in mice [2]	Tumor in human beings
Acid phosphatase	270	250–1,400 (*69*)
Alkaline phosphatase	262	[3] 1,000 (*66*)
Do		[4] 1,000–17,400 (*66*)
Do		170–128,000 (*69*)

[1] For explanation of figures, see table 1, footnote 1.
[2] Transplanted tumors in C3H mice, unpublished data.
[3] Osteolytic type.
[4] Osteoblastic type.

Alkaline phosphatase has long been known to occur in large quantities at sites of ossification in embryos and in children (*65*, *68*). It has been suggested that this enzyme hydrolyzes the phosphoric esters in the blood to bring about a local increase in the concentration of the phosphate ions thereby leading to excess of the solubility product of the calcium phosphates which enter into the structure of bone (*70*). If phosphatase activity runs parallel to osteogenesis it would be expected that the osteogenic tumors would be high in this enzyme, and the data in table 5 reveal that this is indeed the case. It seems probable that phosphatase is synthesized by the osteoblasts.

The control tissues for the tumors mentioned in table 5 were the long bones of adult mice and of human beings. If the growing bones of young mice or children had been selected for the control tissues, the ratio of tumor to control phosphatase would certainly be smaller. Nevertheless, the data reveal a marked increase in the bone phosphatase of the tumors, particularly of the osteoblastic type. The plasma phosphatase in patients with bone tumors also tends to increase markedly, and this will be discussed elsewhere.

It is of interest to consider the marked similarity in the great increase of phospha-

[1] *Greenstein, J. P.: Unpublished data.*

tase activity in osteogenic sarcoma and in the bones at sites of osteoplastic metastases from primary carcinoma of the prostate. It has been suggested that certain metastases stimulate the production of alkaline phosphatase by osteogenic cells at the site of the metastasis (*64*). Whether the initiation of osteogenesis is a function of the metastatic tumor cell or whether the latter carries with it all the materials ready-made for osteogenesis, including a large amount of alkaline phosphatase, can perhaps be better answered by study of the primary prostatic carcinoma or perhaps by metastases to nonosseus tissues naturally low in alkaline phosphatase. This question has been considered in the preceding section.

General Considerations

A survey of the data reveals that no simple generalization which covers the enzymatic behavior of all or even any one group of tumors can be given at the present time. Such statements as that of Edlbacher and Merz (*71*) that all tumors are characterized by a higher arginase content than normal tissues have been based on inadequate material and faulty comparison. Little advantage accrues to an oversimplification of a problem, the very complexity of which offers a fertile field of challenge.

When a normal tissue becomes neoplastic, certain enzyme systems may decrease in activity, others may increase, and still others may not change. The direction and order of magnitude of the change depend on the species of animal, the strain in any given species, as well as the kind of tumor under consideration. When comparison is made between several tumors arising differently in different species and strains from the same kind of tissue, such as the hepatomas, it is found that these hepatomas in mice may for certain enzyme systems be equal in activity; for other enzyme systems they may be equal in each strain but may differ from strain to strain, and for other systems may differ distinctly from tumor to tumor. Arginase and riboflavin belong to the first category; catalase, xanthine dehydrogenase, thymonucleodepolymerase, and to a certain extent acid and alkaline phosphatase, to the second category; and amylase to the third. These relations hold for mouse hepatomas. For mammary tumors in mice, not only arginase but also catalase and the phosphatases belong to the first category. For the lymphomas in mice, arginase belongs to the third category. Alkaline phosphatase is highly active in spontaneous tumors in mice but is present in negligible amounts in the breast carcinoma of human beings (*66*). The normal prostate of man is rich in acid phosphatase, but in most species of animals the prostate gland contains a very small amount of this enzyme. Tumors in man which are strongly neoplastic cause in general a distinct rise in the acid and alkaline phosphatase of the serum; in rats that bear the *p*-dimethylaminoazobenzene-induced hepatoma containing large amounts of alkaline phosphatase, the phosphatase activity of the serum is normal (*57*).

The behavior of the enzymes in a tumor in one species may not necessarily be the same as that of the enzymes in a tumor arising from the same kind of tissue in another species. Nevertheless, the picture given by all the enzymes in hepatomas, for example, is more nearly the same in rats and in several strains of mice than it is like that given by all the enzymes in mammary tumors or in lymphomas, for instance. It is necessary in the enzymatic characterization of tissues, whether neoplastic or otherwise, to have an enzyme "spectrum" or pattern in view. The study of one enzyme is insufficient;

many enzymes must be studied; only in this way can the tissue be described and the possible aberrations of individual enzyme systems be noted. A complete assay of such enzyme spectra in tumors arising from the same tissue in several different species is not yet available.

The range in activity for each of the enzymes so far observed among hepatomas, lymphomas, and mammary, gastric, and intestinal tumors in mice, taken together, is much narrower than that for each of the same enzymes in the normal tissues of origin of these tumors taken together. In general, the range of enzymic activity is much greater among normal tissues than among tumors. It is as if the tumors approached a common metabolic level [2] (*34*). The leveling effect is obviously owing to the frequent loss in specific function when the normal tissue becomes neoplastic. This is most strikingly observed in the tissues above-mentioned, when the neoplastic change involves the severe decrease in such highly active systems as hepatic arginase and catalase, lymphatic depolymerase, and intestinal alkaline phosphatase, etc. At least for mouse tumors, it would appear that enzymatically the tumors are more nearly like each other than they are like normal tissues or than normal tissues are like each other.

COMPARISON OF THE ACTIVITY OF ENZYMES AND CONCENTRATIONS OF CERTAIN COMPONENTS OF THE TISSUES OF NORMAL AND OF TUMOR-BEARING ANIMALS

Tissues Other Than Blood or Serum

The purpose of these studies is to observe whether any effect is elicited in the tissues of tumor-bearing animals at sites relatively far removed from the tumor. Whatever effect the tumor produces it can only do so in either of two ways: (1) By the liberation into the blood stream of some material elaborated by the tumor; or (2) by the abstraction by the tumor of some component in the blood stream which is necessary for the maintenance or proper function of the distant tissue. Little or no evidence is available at present to decide between the alternatives for any one case; it is more important to collect the evidence for the presence of systemic effects elicited by tumors and to postpone possible explanations and interpretations for future consideration.

What is also important is to relate the systemic effect observed directly with the growth of the tumor so as to avoid the possibility that the effects produced may be those attributable to nonspecified causes such as cachexia or toxemia of infection. For this purpose it is necessary to use animals in which the tumors are free from ulceration or infection, to observe whatever systemic effect is produced at intervals during the growth of the tumor and within the period when the animals appear to be healthy and vigorous, and finally to study several enzyme systems and other components in the various tissues. The last-mentioned recommendation is strongly urged because whereas a general toxic condition might depress the activity of all or most of the enzyme systems of a tissue, a specific effect elicited by the tumor might be directed only toward certain catalytic systems. Few studies in the literature meet all these criteria; many meet them in part, and the data selected, which represent the maximum systemic effect produced, are given in table 6.

Inspection of table 6 reveals that in many tissues of the tumor-bearing animals profound changes from the normal values are apparent in certain enzyme systems

[2] *Greenstein, J. P.: Unpublished data.*

and other components whereas no changes are apparent in other systems even in the same tissue.

Liver

In rats bearing transplanted hepatomas or Jensen sarcomas or in mice bearing various transplanted tumors, the activity of the liver catalase is markedly depressed below normal, but the activity of xanthine dehydrogenase and of acid and alkaline phosphatase is normal. The fact that the activity of xanthine dehydrogenase and *d*-amino acid oxidase is normal is of interest because of the lowered value for riboflavin concentration. Evidently there is still

TABLE 6.—*Activity of enzymes and concentration of components of tissues other than blood or serum of normal and of tumor-bearing animals* [1]

Enzyme or component	Tissues of tumor bearing animals				
	Liver	Kidney	Spleen	Muscle	Adrenals
Catalase	[2] 10 (73) [3] 10 (74) [5] 10–40 (75) [4] 6 (75) [7] 25 (76)	[3] 80 (74) [4] 50 (74) [6] 20 (74)			
Arginase	[2] 60 (14) [9] 61 (79) [10] 100 (14) [3] 100 (80)	[7] 28 (77)		[8] > 100 (78)	
Xanthine dehydrogenase	[2] 100 (44) [10] 100 (55)				
d-Amino acid oxidase	[2] 95 (15)				
Riboflavin	[11] 60 (19) [2] 72 (16)				
Cytochrome oxidase	[2] 96 (15)				
Vitamin A	[12] 97 (81)				
Cathepsin	[3] 124 (82) [3] 148 (80) [13] 143 (80) [7] 131 (83)	[7] 104 (83)	[7] 171 (83)		
Glyoxalase	[9] 100 (84)	[9] 100 (84)	[9] 100 (84)	[9] 100 (84)	
Thymonucleodepolymerase	[2] 100		[2] 100 (56)		
Nuclease	[3] 100 (85)			[3] 50, 000 (85) [3] 9, 000 (86) [8] 4 000 (86) [7] 400 (87)	
Acid phosphatase	[2 3 4] 100 (57)	[2 3 4] 100 (57)	[2 3 4] 100 (57)	[2 3 4] 100 (57)	
Alkaline phosphatase	[2 3 4] 100 (57)	[2 3 4] 100 (57)	[2 3 4] 100 (57)	[2 3 4] 100 (57)	
Esterase	[3] 36 (87) [14] 85 (88)	[3] 66 (87)			
Lipase	[3] 36 (89) [14] 91 (88)				
Phospholipid	[15] 50–72 (90)				
Free cholesterol	[2] 85 (91)	[2] 73 (91)	[2] 87 (91)	[2] 110 (91)	[2] 75 (91)
Cholesterol esters	[2] 61 (91)	[2] 143 (91)	[2] 82 (91)	[2] 87 (91)	[2] 68 (91)
Fatty acids	[2] 63 (91)	[2] 161 (91)	[2] 91 (91)	[2] 41 (91)	[2] 10 (91)
Water content	[2] 57 (91)	[2] 42 (91)	[2] 47 (91)	[2] 59 (91)	[2] 68 (91)
Copper	[2] 101 (91) [2] 105 (16) [2] 160 (40) [3] 160 (40) [4] 130 (40) [6] 113 (40)	[2] 102 (91) [6] 84 (40) [3] 55 (40)	[2] 101 (91)	[2] 102 (91)	[2] 104 (91)

[1] Value for normal tissue is taken as 100, and value of tissue from tumor-bearing animals is given on this basis. References to literature are given in parentheses.
[2] Rats bearing transplanted hepatoma.
[3] Rats bearing transplanted Jensen sarcoma.
[4] Mice of various strains bearing the rapidly growing transplanted sarcoma 37 or sarcoma 180.
[5] Mice of various strains bearing transplanted pulmonary, gastric, brain, hepatic, melanotic, and lymphatic tumors, and spontaneous mammary tumors.
[6] C3H mice bearing spontaneous mammary tumors.
[7] Mice bearing the Ehrlich carcinoma.
[8] Rats bearing the transplanted Flexner-Jobling sarcoma.
[9] Rats bearing transplanted Philadelphia sarcoma 1.
[10] Mice of various strains bearing transplanted hepatic tumors.
[11] Noncancerous portion of liver with hepatoma induced by *p*-dimethylaminoazobenzene.
[12] Human beings with gastro-intestinal tumors.
[13] Horse with melanosarcoma.
[14] Rats bearing Walker carcinosarcoma.
[15] Mice bearing transplanted sarcomas, lymphomas, or mammary carcinomas.

enough of the riboflavin present in the liver to account for the normal activity of these two enzymes. Certain other components, cathepsin and copper, appear to be augmented in the livers of tumor bearing animals. The livers as well as other tissues of the tumor-bearing animals contain a somewhat higher water content than do the same tissues in normal animals.

Certain species differences are apparent, i. e., arginase is decreased in the livers of tumor-bearing rats but not in the livers of tumor-bearing mice. Certain differences within the species are also apparent, for the hepatic esterase and lipase activity of rats bearing the Jensen sarcoma appear to be much more depressed than in rats bearing the Walker tumor. The relative rate of tumor growth may explain this difference in part.

The enzyme system most thoroughly studied in the tissues of tumor-bearing animals has been catalase (*74*). Nearly invariably, the activity of this enzyme in the livers and kidneys of rats and of mice bearing a wide variety of tumors is lowered considerably below normal. One of the earliest reports on the systemic effect of tumors was made by Brahn (*92*) who observed very low liver catalase values in human beings who had died as a result of various forms of cancer. The low liver catalase activity in various species of tumor-bearing animals is also frequently paralleled by low blood hemoglobin concentrations. Both catalase and hemoglobin are proteins which contain the same hematoporphyrin nucleus. There does not appear to be any direct effect of the tumor on the liver catalase molecule, for incubation *in vitro* of the extracts or slices of tumors with extracts or slices of liver or with solutions of crystalline catalase does not alter the activity of the catalase in any way.[3] There is likewise no evidence for the presence of a dissociable inhibitor for catalase in the liver of tumor-bearing animals.[4] It may be that the effect of the tumor on the liver catalase activity is caused by an interference with the synthesis of the hematoporphyrin nucleus.

Kidney, Spleen, and Adrenals

Catalase, arginase, esterase, and copper appear to be considerably reduced below the normal level in the kidneys of tumor-bearing animals. On the other hand, free and esterified cholesterol are markedly increased. The extent of the drop in the kidney catalase activity is not so great as in the liver, and in most tumor-bearing animals the kidney catalase activity is much greater per gram of tissue than the liver catalase.

The catheptic activity of the spleen like that of the liver appears to be definitely increased in tumor-bearing animals. The spleen frequently hypertrophies in tumor-bearing animals. Other enzymes are little affected.

The content of various fats is lowered considerably below the normal in the adrenal glands of tumor-bearing rats.

Muscle

A remarkable finding is reported concerning the practical induction of nuclease activity in the striated muscle of tumor-bearing rats and mice (*85*, *86*). This enzyme, the activity of which is followed by the splitting of phosphoric acid from thymonucleic or ribosenucleic acid, is either absent or present in negligible amounts in the muscles of normal animals. The extent of its appearance in the muscles of the tumor-bearing animals is unusual. The original observations of Edlbacher and Kutscher (*85*) were confirmed and extended by Wienbeck (*86*) in another laboratory. Further extensions of this observation are clearly desirable.

[3] [4] *Greenstein, J. P.: See pp. 397 to 404 of this Journal.*

A definite increase in the arginase activity of the muscles of tumor-bearing animals has also been reported (*78*). The data in the original paper are, however, very brief.

Relation of Growth Rate of Tumor to Systemic Effect

If the tumor is directly or indirectly responsible for producing an effect on a distant tissue of the tumor-bearing animal, it might be expected that this effect would increase in intensity with progressive growth of the tumor. A few of the investigators of the systemic effect considered this possibility. Edlbacher and Kutscher (*85*) pointed out that the degree of induction of nuclease activity in the muscle tissue of tumor-bearing animals was proportional to the growth rate of the tumor. Fujiwara (*77*), Green (*87*), and others (*74, 93*) made similar observations for other enzyme systems in other tissues in tumor-bearing animals. Greenstein and Andervont (*75*) observed that sarcoma 37 cutaneously inoculated into Y, C, and dilute brown mice grew rapidly and that the liver catalase activity of these animals dropped rapidly during growth of the tumor and at about the same rate in each strain. When the same tumor was inoculated subcutaneously into the tails of mice of these strains, it grew in all strains at about half the rate as when cutaneously implanted, and the rate of fall of the liver catalase activity was roughly half that observed in animals with tumors implanted cutaneously. It was emphasized recently (*74*) that the degree of the systemic effect observed has no meaning unless the corresponding weight and age of the tumor is known. In any event, all observations indicate that the systemic effects elicited by the tumor generally parallel in degree the growth of the tumor. This is an important fact. For one thing, the available evidence indicates that the systemic effect changes in degree but not in kind during growth of the tumor, and hence the property of the tumor which is responsible for the systemic effect changes quantitatively but not qualitatively. The tumor, at least as far as this property is concerned, remains constant during its existence.

When a tumor grows extremely slowly, it is often unlikely that a systemic effect will be evident. This has been observed in the case of certain slowly growing mouse tumors (*75*). It is possible that in the tumor-bearing animals a competition exists between the rate of the systemic effect produced in a tissue by the presence of a distant tumor and the rate of repair by that tissue. In animals with rapidly growing tumors the former rate predominates, and conversely the latter rate might predominate in animals with the very slowly growing tumors.

Finally, it is possible that even in animals bearing rapidly growing tumors some systemic effect may be absent. This has been observed in the case of C57 Black mice bearing sarcoma 37 (*75*). In this strain the enzymatic activity of the livers of the normal animals is much below that of the livers of many other mouse strains, and this might be an explanation for the anomalous behavior. The enzymatic activity of the tissues of different strains of normal mice may vary considerably, and it is evident that this factor must be taken into account. The normal base level of activity of the tissue enzymes of available strains should be known and used in any work of this kind.

Reversibility of the Systemic Effect

If the presence of the tumor is directly responsible for the systemic effect produced in distant tissues, it might be expected that complete removal of the tumor from the tissues would result in the disappearance of the systemic effect and in

the restoration of the distant tissue to its normal condition. This expectation has been fulfilled (*73*, *75*). For permanent restoration the absence of metastases is taken for granted.

The catalase activity of livers of rats bearing the transplanted hepatoma 31 is very much lower than that of livers of normal rats (*73*). This tumor is encapsulated and readily removed from the animal under ether anesthesia. Within 48 hours following such an operation the liver of the animal has a normal catalase value. If a new tumor is implanted into the same animal the liver catalase activity again decreases and is again restored to normal by a second operative removal of the tumor.

The catalase activity of the livers of C, Y, and dilute brown mice all bearing the intracutaneously transplanted sarcoma 37 is very much lower than that of the livers of normal animals (*75*). Under these conditions of growth the tumor cannot be completely removed, for it readily invades the surrounding tissues. Incomplete removal of the tumor from these animals causes a temporary rise in the liver catalase activity which is roughly proportional to the amount of tumor removed and which quickly drops again as the tumor continues to grow. The technique developed by Andervont for immunity studies (*94*) can be used to remove tumors completely. Sarcoma 37 may be implanted in the tails of mice where it grows at a somewhat slower rate than it does in the abdominal skin. The drop in the liver catalase activity of the animals with the growth of such caudal implants is also proportionately slower. When the tails are amputated, the tumors are completely removed from the animal. Within 3 days after this operation, the liver catalase activity of the mice is restored to the normal level which is maintained thereafter (*75*).

Physical removal of the tumor is not the only evidence of reversibility of the systemic effect. Transplanted sarcoma 37 grows progressively in I strain mice for about 2 to 3 weeks after implantation when it starts to regress spontaneously and after about 5 to 6 weeks it disappears completely. The liver catalase activity of strain I mice bearing this tumor drops with growth of the tumor and then rises with regression until it reaches the normal level again with disappearance of the tumor (*75*). This case illustrates the necessity of studying systemic effects in animals bearing progressively growing tumors.

The reversibility of systemic effects elicited in human serum by the presence of tumors has been often demonstrated following removal of the tumors. These cases will be reviewed in the section on serum.

Blood and Serum

That marked and distinct changes in the character of the blood plasma as a result of cancerous growth might serve as a practical diagnostic test was early recognized by clinical workers in the field. Particularly in the case of suspected tumors in difficultly accessible sites, the blood might serve as a window through which the progressive development of the disease or its treatment could be made visible. This naturally presupposes the ability to distinguish such alterations as are revealed from normal individual variations and from changes caused by pathologic conditions other than cancer. However, even changes not specific to cancer might at the cost of their practical value possess an interest of their own. The medical literature, chiefly that of Central Europe, has frequently been the source of numerous announcements concerning serochemical diagnostic reactions for cancer. Woodhouse (*95*) and Rosen-

thal (*96*) have recently summarized most of them. Many appear to involve unusual reactions and to lack any basis in accepted physiology. Others appear to be based upon the conception that the organisms can produce an antibody reaction to malignant cells (Abderhalden, Freund-Kaminer, Fuchs), which may be observed by the degree of proteolytic activity of the suspected serum acting upon serum protein substrates of various origin. A vast literature, both pro and con, has developed about tests based on this concept, and it seems necessary only to consider the experimental techniques involved in these tests. To take only one illustration from the frequently advocated Fuchs test: the preparation of the specific serum substrates by Fuchs and by his followers involves the precipitation of the proteins from serum by adding 11 times the volume of 2.5 percent trichloracetic acid and by subsequent frequent washing with this acid. One might wonder if the proteins so obtained retain any specific character after such treatment. After this, the extraordinarily involved technique apparently necessary to pick up minute differences by titration of the digests appears to be rather a test of the experimenter's patience. It is possible that the concepts upon which these tests are based may be well-founded and capable of experimental proof. Until such time, however, that these tests are performed according to practices that are accepted by the experienced biochemist any final assessment of their value must be postponed.

Other recent types of serochemical diagnosis appear to be the result of an overready acceptance of the Kögl hypothesis concerning the presence of the unnatural *d*-amino acid optical isomers in tumors. The assumption is that if these isomers exist in peptide combination in the tumor there must be peptidase present in the tumor capable of catalyzing the hydrolysis of such peptides; during development of the tumor some of these enzymes would presumably "leak" into the blood stream. The results have been highly indecisive; the data for the most part have been summarized by Maver, Johnson, and Thompson (*97*), who have questioned the specificity of the method. It has long been known that many normal tissues are capable of splitting peptides involving the unnatural amino acid isomers (*98*). Furthermore, one may perhaps doubt the evidence which rests upon the practice of conducting digestions at an elevated temperature of crude serum for the length of time (2–5 days) apparently needed in order to observe such reactions as have been reported. With the discrediting of the Kögl hypothesis any basis for the postulated serum reaction vanished.

To the reader who has studied the vast literature of cancer serochemistry, the subject appears to be more a sequence of psychologic than chemical phenomena. The urgency of the immediate task has perhaps been the frequent cause of confusion. Perhaps the advisable approach would be not to insist at the very start that a rigorous, practical, and specific test for malignancy be established, but that the readily observed changes in the blood or sera of tumor-bearing individuals be more closely examined even if such changes appear to occur in conditions other than cancer. On further closer scrutiny such changes in the cancerous individual might be distinguished from those in the noncancerous individual. Even in those cases in which this may be unsuccessful, such information has an independent interest. The reviewer has therefore selected evidence that appears to be sufficiently authentic to illustrate systemic effects elicited in the blood of the tumor-bearing host, (1) a progressive depression in the

blood hemoglobin level of many species of animals during the growth of many kinds of tumors, (2) a marked decrease in the serum esterase and lipase activity as a result of tumor growth in different species, and (3) the marked rise in serum phosphatase activity, particularly acid phosphatase, in prostatic carcinoma in man. Only the last-mentioned appears to be specific for cancer, but all three are closely associated with the growth of the tumors.

Blood Hemoglobin

Many investigators have pointed out that anemia generally accompanies cancer. The pertinent literature has been summarized by Taylor and Pollack (*99*). The anemic condition appears to be brought about in many species of animals bearing many types of malignant growths. Reliable evidence that there is a direct relation between the hemoglobin level and the course of growth of implanted as well as induced tumors in animals has been reported by Taylor and Pollack (*99*). The hemoglobin level decreases progressively to very low levels during the period of growth of the implanted tumor, during the precancerous stage in animals treated with carcinogens, and during the growth of the induced tumor. Strong and Frances (*100*) show that the hemoglobin level of mice of high spontaneous tumor incidence falls off markedly even before the tumorous growth appears, in contrast with strains of low incidence in which this effect does not appear. It is suggestive, as mentioned previously, that one of the most marked systemic effects produced in the tissue of the tumor-bearing animals and one which, like the hemoglobin lowering, appears to occur in all species studied, is the lowering of catalase activity. Hemoglobin and catalase contain the same prosthetic group, the hematoporphyrin nucleus, and it may be that the presence of the tumor interferes in some manner with the synthesis of this nucleus. Such possible interference by the tumor is, however, not equally distributed in all tissues; the liver catalase activity of tumor-bearing animals is depressed much more than is the kidney catalase, whereas the blood catalase is very little affected (*74*). This variation illustrates the relative independence of catalase and of hemoglobin within the erythrocyte and suggests the possibility that these two components enter the red cell from different sites.

Blood and Serum Esterase and Lipase

Several independent investigators (*87*, *88*, *89*, *101*, *102*, *103*, *104*) have clearly demonstrated that the activity of blood and serum lipase and esterase in tumor-bearing animals progressively decreases to very low levels during growth of the tumor. In rats with implanted tumors the activity may be reduced to a half to one-quarter of the normal activity, and the change begins at an early stage of the tumor growth. In animals in which the tumor begins to regress spontaneously, the serum lipase activity rises; and when the tumor has disappeared, the enzyme activity reaches the normal level (*87*). The parallel behavior of the serum lipase and tumor growth or regression is proof that the extent of the enzyme activity is closely associated with the cancerous state of the host. In tumor-bearing animals the esterase activity of other tissues such as the liver and kidney is considerably lower than normal (table 6).

Serum Phosphatase

Kay (*105*) pointed out in 1930 that the plasma phosphatase (at pH 7.6) was considerably elevated above normal in generalized diseases of bone. The rise in plasma phosphatase activity appears to be a reflection of the tendency to bone forma-

tion and thus occurs normally in growing children as well as in such diseases as rickets, hyperparathyroidism, and osteogenic sarcoma. In the case of carcinoma of various tissues which involve osteoplastic metastases the alkaline phosphatase activity in the blood is also considerably elevated (*64, 66, 106*). Woodard and Craver (*107*) have pointed out that the activity of the serum phosphatase is frequently high in lymphoid diseases long before bone changes become evident, and they suggest that the bone changes may occur more frequently than was formerly suspected. It seems clear at the present time that an elevation in the serum alkaline phosphatase activity, in the absence of jaundice, is evidence for the presence of osteoblastic activity in the host brought about by a variety of conditions. It remains true, however, that the highest values reported as a result of any one of these conditions occur in cases of osteogenic sarcoma (*66, 108*). Removal of the osteogenic sarcoma by operation is followed by a drop of the plasma phosphatase to the normal level, and recurrence of the tumor is followed by a second rise (*66*). The effect of the tumor is thus reversible and similar to the experimental studies on the rat hepatoma in the section entitled "Reversibility of the Systemic Effect."

Phosphatase activity in all tissues studied possesses two maxima at different pH levels, one at about pH 9 and the other at about pH 5. Perlmann and Ferry (*109*) have recently adduced evidence that the activity demonstrated at each of these levels is caused by a separate enzyme. The normal prostate gland in man is rich in the acid phosphatase (*62, 63*). In primary carcinoma of the prostate with osteoplastic skeletal metastases, the Gutmans (*63*) found not only an elevated alkaline but also an elevated acid serum phosphatase. This is an important observation for it enables the clinician to differentiate the general osteoplastic character of the high serum alkaline phosphatase from the high acid serum phosphatase which appears to be specific for prostatic carcinoma (*64, 110*). The observation of the Gutmans has been amply confirmed by many subsequent investigators (*65, 111, 112, 113, 114, 115, 116*). Indeed, the level of acid serum phosphates has with some confidence been used as a prognostic sign during the course of the disease. In general, the higher this value the more unfavorable the prognosis has been found to be.

In prostatic cancer with marked elevation of the serum acid phosphatase, Huggins and Hodges (*113*) found that castration or injection of large amounts of estrogen produced in general a sharp reduction of this enzyme to or toward the normal range. Cessation of administration of stilbestrol was followed by a rise in the enzyme activity, and subsequent administration was again followed by a return to the normal level. In most cases castration or administration of stilbestrol was followed by clinical improvement (*115*). The serum acid phosphatase level is thus associated with the growth of the tumor and agrees with the other systemic effects described herein, such as the changes in liver catalase, blood hemoglobin, and serum lipase. In the case of the prostatic carcinoma, however, the relation between the tumor and the enzyme may be mediated by hormone control. The activity of the prostatic tissue as reflected by the rise of the acid serum phosphatase apparently depends in part upon the degree of androgenic hormone activity. This enzyme varies according to effect of androgen control of the prostatic epithelium. The changes in the alkaline serum phosphatase activity bear little relation to the changes in the acid phosphatase since the former are apparently due only to the level of osteo-

blastic cells. Such changes as occur in the alkaline serum phosphatase will be of a secondary nature only. If the carcinomatous prostatic epithelium is inhibited by estrogens it might be expected that it would be activated by androgen injections, and this is indeed the case. A rise in the serum acid phosphatase above the pre-injection level was observed (*113*). Bilateral orchidectomy in dogs resulted in no change in the level of the serum phosphatases.

Attempts to influence the acid phosphatase activity of the prostate glands of both mature and immature rats by injection of various sex hormones were unsuccessful (*72*). However, further studies in experimental animals of the hormonal control of tumors of the sexual and accessory tissues await the production of such tumors in animals of appropriate strains. Such techniques as described by Shimkin, Grady, and Andervont (*117*) offer much promise in this direction.

General Considerations

The marked changes from the normal values of the tissue components in tumor-bearing animals have been described as systemic effects elicited by the presence of the tumor. The fundamental criterion on which these effects rest may be expressed as follows: the effect must be progressive with the growth of the tumor, increasing as the tumor grows and decreasing if the tumor regresses or is operatively removed. According to this criterion the effect is directly related to the existence of the tumor, but so far we have little evidence as to whether such an effect is specifically related to the tumor. Nevertheless, the fact that pronounced effects occur in tissues relatively far removed from the site of the tumor lends weight to the contemporary viewpoint that cancer is not a local but a generalized disease.

Many other pathologic states of the animal are recognized by systemic effects which rise in intensity with the progress of the disease and subside with its fall, e. g., diabetes, certain psychotic states, thyroidism, etc. In this respect cancer is not a unique disease. But just as the effects elicited by diabetes may be distinguished from those observed in pituitary disease, so it might be possible to relate specifically certain systemic effects in the tumor-bearing animal to the growth of the tumor. Whatever success has attended the former observations has been largely owing to the ability to link the effects noted with the physiology of the affected tissue. We know very little of the physiology of tumors in general. The decrease in activity of catalase and esterase and the increase of phosphatase and nuclease in the tissue of tumor-bearing animals may or may not be related to each other. If more were known about the metabolism of the tumor, the bearing of these phenomena on the growth of the tumor might be explicable. The study of systemic enzymatic effects elicited in the tumor-bearing animal by the presence of the tumor constitutes an indirect approach to the cancer problem in contrast with the direct approach offered by the study of the tumor itself. Each approach supplements the other, and both are necessary for the elucidation of this complex problem.

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